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(74) Agent: A.P.T. PATENT AND TRADE MARK ATTOR-
NEYS; P.O. Box 222, Mitcham, South Australia 5062
(AU).

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(71) Applicants (for all designated States except US): COM-
MONWEALTH SCIENTIFIC AND INDUSTRIAL
RESEARCH ORGANISATION [AU/AU]; Limestone
Avenue, Campbell, Australian Capital Territory 2612
(AU). GF GROUP SERVICES PTY LTD [AU/AU]; 75
Talavera Road, Macquarie Park, NSW 2113 (AU).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BIOGEMMA S A
S [FR/FR]; 1, rue Edouard Colonne, F-75001 Paris (FR).
REGINA, Ahmed [IN/AU]; 4 Nangi Place, Ngunnawal,
Australian Capital Territory 2913 (AU). RAHMAN,
Sadequr [AU/AU]; 36 Temperley Street, Nicholls, Aus-
tralian Capital Territory 2913 (AU). MORELL, Matthew,
Kennedy [AU/AU]; 13 Wolgal Place, Aranda, Australian
Capital Territory 2614 (AU). LI, Zhongyi [CN/AU]; 51
Campaspe Circuit, Kaleen, Australian Capital Territory
2617 (AU).

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(54) Title: WHEAT WITH ALTERED BRANCHING ENZYME ACTIVITY AND STARCH AND STARCH CONTAINING
PRODUCTS DERIVED THEREFROM

(57) Abstract: Wheat having a reduced level of SBEIIa activity, that may have a relative high amylose content. Wheat having a
mutant SBEIIa gene in the A genome. The wheat might additionally have reduced levels of SBEIIb activity. The wheat grain of
this invention can be of a non-shrunken phenotype despite a lesion in the amylopectin synthesis pathway, and may also have a high
relative amylose content.

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WHEAT WITH ALTERED BRANCHING ENZYME ACTIVITY AND STARCH AND STARCH CONTAINING PRODUCTS DERIVED THEREFROM

5 FIELD OF THE INVENTION

This invention relates to a wheat plant having kernel starch with a high relative amylose content. The invention also relates to wheat with a reduced starch branching enzyme IIa (SBEIIa) activity in the endosperm and methods of obtaining such plants. The invention also relates to grain and starch and food and non-food products obtained therefrom.

10

BACKGROUND OF THE INVENTION

In cereals, starch makes up approximately 45-65% of the weight of the mature grain. The starch is composed of two types of molecule, amylose and amylopectin. Amylose is an essentially linear molecule composed of α -1,4 linked glucosidic chains, while
15 amylopectin is highly branched with α -1,6 glucosidic bonds linking linear chains.

The synthesis of starch in the endosperm of higher plants is carried out by a suite of enzymes that catalyse four key steps. Firstly, ADP-glucose pyrophosphorylase activates the monomer precursor of starch through the synthesis of ADP-glucose from G-1-P
20 and ATP. Secondly, the activated glucosyl donor, ADP-glucose, is transferred to the non-reducing end of a pre-existing α 1-4 linkage by starch synthases. Thirdly, starch branching enzymes introduce branch points through the cleavage of a region of α -1,4 linked glucan followed by transfer of the cleaved chain to an acceptor chain, forming a new α -1,6 linkage. Starch branching enzymes are the only enzymes that can introduce
25 the α -1,6 linkages into α -polyglucans and therefore play an essential role in the formation of amylopectin. Finally, starch debranching enzymes remove some of the branch linkages although the mechanism through which they act is unresolved (Myers *et al.*, 2000).

30 While it is clear that at least these four activities are required for normal starch granule synthesis in higher plants, multiple isoforms of each of the four activities are found in the endosperm of higher plants and specific roles have been proposed for individual isoforms on the basis of mutational analysis (Wang *et al.*, 1998, Buleon *et al.*, 1998) or through the modification of gene expression levels using transgenic approaches (Abel *et al.*, 1996, Jobling *et al.*, 1999, Scwall *et al.*, 2000). However, the precise contributions
35 of each isoform of each activity to starch biosynthesis are still not known, and it is not known whether these contributions differ markedly between species. In the cereal

endosperm, two isoforms of ADP-glucose pyrophosphorylase are present, one form within the amyloplast, and one form in the cytoplasm (Denyer *et al.*, 1996, Thorbjornsen *et al.*, 1996). Each form is composed of two subunit types. The shrunken (*sh2*) and brittle (*bt2*) mutants in maize represent lesions in large and small subunits respectively (Giroux and Hannah, 1994). Four classes of starch synthase are found in the cereal endosperm, an isoform exclusively localised within the starch granule, granule-bound starch synthase (GBSS), two forms that are partitioned between the granule and the soluble fraction (SSI, Li *et al.*, 1999a, SSII, Li *et al.*, 1999b) and a fourth form that is entirely located in the soluble fraction, SSIII (Cao *et al.*, 2000, Li *et al.*, 1999b, Li *et al.*, 2000). GBSS has been shown to be essential for amylose synthesis (Shure *et al.*, 1983), and mutations in SSII and SSIII have been shown to alter amylopectin structure (Gao *et al.*, 1998, Craig *et al.*, 1998). No mutations defining a role for SSI activity have been described.

Three forms of branching enzyme are expressed in the cereal endosperm, branching enzyme I (SBEI), branching enzyme IIa (SBEIIa) and branching enzyme IIb (SBEIIb) (Hedman and Boyer, 1982, Boyer and Preiss, 1978, Mizuno *et al.*, 1992, Sun *et al.*, 1997). Genomic and cDNA sequences have been characterized for rice (Nakamura and Yamanouchi, 1992), maize (Baba *et al.*, 1991; Fisher *et al.*, 1993; Gao *et al.*, 1997) and wheat (Repellin *et al.*, 1997; Nair *et al.*, 1997; Rahman *et al.*, 1997). Sequence alignment reveals a high degree of sequence similarity at both the nucleotide and amino acid levels and allows the grouping into the SBEI, SBEIIa and SBEIIb classes. SBEIIa and SBEIIb generally exhibit around 80% sequence identity to each other, particularly in the central regions of the genes. SBEIIa and SBEIIb may also be distinguished by their expression patterns. SBEIIb is generally specifically expressed in endosperm while SBEIIa is present in every tissue of the plant.

In wheat endosperm, SBEI (Morell *et al.*, 1997) is found exclusively in the soluble fraction, while SBEIIa and SBEIIb are found in both soluble and starch-granule associated fractions (Rahman *et al.*, 1995). In maize and rice, high amylose phenotypes have been shown to result from lesions in the *SBEIIb* gene, also known as the *amylose extender* (*ae*) gene (Boyer and Preiss, 1981, Mizuno *et al.*, 1993; Nishi *et al.*, 2001). In these *SBEIIb* mutants, endosperm starch grains showed an abnormal morphology, amylose content was significantly elevated, the branch frequency of the residual amylopectin was reduced and the proportion of short chains (<DP17, especially DP8-12) was lower. Moreover, the gelatinisation temperature of the starch was increased. In addition, there was a significant pool of material that was defined as "intermediate"

- between amylose and amylopectin (Boyer *et al.*, 1980, Takeda, *et al.*, 1993b). In contrast, maize plants mutant in the *SBEIIa* gene due to a mutator (Mu) insertional element and consequently lacking in SBEIIa protein expression were indistinguishable from wild-type plants in the branching of endosperm starch (Blauth *et al.*, 2001),
5 although they were altered in leaf starch. Similarly, rice plants deficient in SBEIIa activity exhibited no significant change in the amylopectin chain profile in endosperm (Nakamura 2002). In both maize and rice, the *SBEIIa* and *SBEIIb* genes are not linked in the genome
- 10 In maize, the *dull1* mutation causes decreased starch content and increased amylose levels in endosperm, with the extent of the change depended on the genetic background, and increased degree of branching in the remaining amylopectin (Shannon and Garwood, 1984). The gene corresponding to the mutation was identified and isolated by a transposon-tagging strategy using the transposon mutator (Mu) and shown to encode
15 the enzyme designated starch synthase II (SSII) (Gao *et al.*, 1998). The enzyme is now recognized as a member of the SSIII family in cereals (Li *et al.*, 2003). Mutant endosperm had reduced levels of SBEIIa activity associated with the *dull1* mutation. No corresponding mutation has been reported in other cereals. It is not known if these findings are relevant to other cereals, for example wheat.
- 20 WO94/09144 suggests the use of sense and antisense genes to alter the natural ratios of starch synthase (SS) and SBE in maize. However, no data are presented to substantiate the proposed molecular strategies and there is no suggestion of specifically reducing the activity of SBEIIa.
- 25 In potato, down regulation of *SBEI* alone causes minimal affects on starch structure (Filipse *et al.*, 1996), although further work identified some qualitative changes (Safford *et al.*, 1998). However, in potato the down regulation of *SBEII* and *SBEI* in combination increased the relative amylose content much more than the down-regulation of *SBEII*
30 alone (Schwall *et al.*, 2000).
- 35 Two types of debranching enzymes are present in higher plants and are defined on the basis of their substrate specificities, isoamylase type debranching enzymes, and pullulanase type debranching enzymes (Myers *et al.*, 2000). Sugary-1 mutations in maize and rice are associated with deficiency of both debranching enzymes (James *et al.*, 1995, Kubo *et al.*, 1999) however the causal mutation maps to the same location as the

isoamylase-type debranching enzyme gene. Representative starch biosynthesis genes that have been cloned from cereals are listed in Table 1.

Table 1. Starch branching enzyme genes characterized from cereals.

Species	SBE isoform	Type of clone	Accession No.	Reference
Maize	SBEI	cDNA	U17897	Fisher et al., 1995
		genomic	AF072724	Kim et al., 1998a
	SBEIIb	cDNA	L08065	Fisher et al., 1993
		genomic	AF072725	Kim et al., 1998
	SBEIIa	cDNA	U65948	Gao et al., 1997
Wheat	SBEII	cDNA	Y11282	Nair et al., 1997
	SBEI	cDNA and genomic	AJ237897 (SBEI gene)	Baga et al., 1999
			AF002821 (SBEI pseudogene)	Rahman et al., 1997,
			AF076680 (SBEI gene)	Rahman et al., 1999
			AF076679 (SBEI cDNA)	
	SBEI	cDNA	Y12320	Repellin et al., 1997
	SBEIIa	cDNA and genomic	AF338432 (cDNA)	Rahman et al., 2001
	SBEIIb	cDNA and genomic	AF338431 (gene)	
				WO 01/62934
	SBEIIb	cDNA		WO 00/15810
Rice	SBEI	cDNA	D10752	Nakamura and Yamanouchi, 1992
	SBEI	genomic	D10838	Kawasaki et al., 1993
	RBE3	cDNA	D16201	Mizuno et al., 1993
Barley	SBEIIa and SBEIIb	cDNA and genomic	AF064563 (SBEIIb gene) AF064561 (SBEIIb cDNA) AF064562 (SBEIIa gene) AF064560 (SBEIIa cDNA)	Sun et al., 1998

Starch is widely used in the food, paper and chemical industries. The physical structure of starch can have an important impact on the nutritional and handling properties of starch for food or non-food or industrial products. Certain characteristics can be taken as an indication of starch structure including the distribution of amylopectin chain length, the degree and type of crystallinity, and properties such as gelatinisation temperature, viscosity and swelling volume. Changes in amylopectin chain length may be an indicator of altered crystallinity, gelatinisation or retrogradation of the amylopectin.

Starch composition, in particular the form called resistant starch which may be associated with high amylose content, has important implications for bowel health, in particular health of the large bowel. Accordingly, high amylose starches have been developed in certain grains such as maize for use in foods as a means of promoting bowel health. The beneficial effects of resistant starch result from the provision of a nutrient to the large bowel wherein the intestinal microflora are given an energy source which is fermented to form *inter alia* short chain fatty acids. These short chain fatty acids provide nutrients for the colonocytes, enhance the uptake of certain nutrients across the large bowel and promote physiological activity of the colon. Generally if resistant starches or other dietary fibre is not provided the colon is metabolically relatively inactive.

Whilst chemically or otherwise modified starches can be utilised in foods that provide functionality not normally afforded by unmodified sources, such processing has a tendency to either alter other components of value or carry the perception of being undesirable due to processes involved in modification. Therefore it is preferable to provide sources of constituents that can be used in unmodified form in foods.

More wheat is produced in the world each year than for any other cereal grain crop. Known variation in wheat starch structure is limited relative to the variation available in maize or rice, in part because the transformation efficiency of wheat has lagged behind that for other cereals, and because of the hexaploid nature of breadwheat. The presence of three genomes in *Triticum aestivum* has a buffering effect by masking mutations in individual genomes, in contrast to the more readily identified mutations in diploid species. Mutants in *SBEIIb*, corresponding to the amylose-extender phenotypes in maize or rice, have not been characterized in wheat. The phenotype conferred by *SBEIIa* or *SBEIIb* mutations in wheat is unknown. Known mutants are for the waxy gene (GBSS, Zhao and Sharp, 1998) and a mutant entirely lacking the SGP-1 protein (Yamamori et al, 2000) which was produced by crossing lines which were lacking the A, B and D genome specific forms of SGP-1 (SSII) protein as assayed by protein electrophoresis. Examination of the SSII null seeds showed that the mutation resulted in alterations in amylopectin structure, deformed starch granules, and an elevated relative amylose content to about 30-37% of the starch, which was an increase of about 8% over the wild-type level (Yamamori et al., 2000). Amylose was measured by colorimetric measurement, amperometric titration (both for iodine binding) and a concanavalin A method. Starch from the SSII null mutant exhibited a decreased gelatinisation

temperature compared to starch from an equivalent, non-mutant plant. Starch content was reduced from 60% in the wild-type to below 50% in the SSII-null grain.

5 WO99/14314 describes the isolation of an *SBEIIa* gene from *Aegilops tauschii*, a diploid plant related to wheat, but did not produce wheat with altered starch.

10 WO 00/15810 describes the cloning of cDNAs for a wheat SBEIIb gene. They did not obtain wheat plants with altered amylose levels and did not teach wheat having starch comprising at least 50% amylose.

WO01/62934 also describes a wheat SBEIIb gene and suggests introducing inhibitors of branching enzyme activity into a wheat plant but does not teach wheat having starch comprising at least 50% amylose.

15 WO 01/32886 characterized a cDNA encoding a form of SBEI in wheat endosperm. The encoded polypeptide was found to be preferentially associated with A-type starch granules. They did not suppress SBEI activity or show altered starch granule morphology or elevated amylose in wheat.

20 Therefore, wheat having starch with a proportion of amylose greater than about 50% is unknown. Although high amylose maize and barley varieties are known, products from these cereals have disadvantages compared to a very high amylose wheat for products where wheat is the preferred cereal, for example in bread, pasta or noodles.

25 Whilst elevated amylose starches of these types are useful, a wheat starch with higher amylose contents is preferred, in particular if associated with improved starch synthesis and other characteristics, for example a reduced need for post-harvest modification. Such starch products are also relatively resistant to digestion and bring a greater health benefit.

30

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and
35 modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

10

Bibliographic details of the publications referred to by the inventors in this specification are collected at the end of the description. The references mentioned herein are hereby incorporated by reference in their entirety. Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

15

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

20

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents Thymidine.

25

SUMMARY OF THE INVENTION

In a first aspect the invention provides a grain obtained from a wheat plant wherein the proportion of amylose in the starch of the grain is at least 50%. The wheat plant may have a reduced level of SBEIIa gene expression SBEIIa enzyme activity or both, preferably a reduced level of both SBEIIa and SBEIIb gene expression, enzyme activity or both. The grain might comprise a genetic variation that is either a mutation of an SBEIIa gene which inhibits SBEIIa gene expression, enzyme activity or both or an introduced nucleic acid which inhibits SBEIIa gene expression, enzyme activity or both. Additionally the grain might comprise similar genetic variation in SBEI. The grain might additionally comprise an altered level of a protein and/or enzyme activity selected from the group consisting of ADP glucose pyrophosphorylase, GBSS, SSI, SSII, SSIII, a

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- debranching enzyme of an isoamylase type and a debranching enzyme of a pullulanase type. The grain might comprise a transgene and the transgene might encode an antisense, co-suppression, ribozyme or duplex RNA molecule. The transgene preferably leads to a reduced level of expression of an mRNA encoding SBEIIa. The grain might
- 5 comprise a mutation in an SBEIIa gene and in one form is a null mutation of the SBEIIa gene in at least one genome and may be a null mutation in two or three of the genomes. The proportion of amylose in the starch of the grain might be at least 40%, 50%, 55%, 60%, 70% or 80%. In another form at least 50% of starch granules within the grain appear non-birefringent when observed under polarized light. The grain may be non-
- 10 shrunken, and might have an average weight of at least 36mg or 40mg. In an alternative form the starch content of the grain when naked is at least 25% (w/w) or at least 35% (w/w), and may be at least 90% of the starch content of wild type grain. The grain may be whole grains, hulled grain, milled, cracked, rolled, pearled, ground or par boiled grain.
- 15 In another form the first aspect the invention provides a grain obtained from a wheat plant, the grain comprising starch and a genetic variation which leads to a reduction in the level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain, which genetic variation comprises a mutation of an SBEIIa gene or an introduced nucleic acid which encodes an inhibitor of SBEIIa gene
- 20 expression; wherein the proportion of amylose in the starch of the grain is at least 30%.
- In a second aspect the invention provides a milled product derived from grain of the first aspect including but not limited to flour, wholemeal, semolina or starch obtained from the grain of the invention, or food products incorporating such flour, wholemeal,
- 25 semolina, or starch or rolled, flaked or extruded products of the grain. The product may include flour, wholemeal, semolina, or starch obtained from the grain of the first aspect of the invention blended with flour, wholemeal, semolina, or starch from another source.
- In a third aspect the invention provides starch granules or starch obtained from grain of the wheat plant of the first aspect. In a specific form of the third aspect, the wheat plant
- 30 additionally has a reduced level of SBEIIa enzyme activity in the endosperm.
- In a fourth aspect the invention might be said to reside in a composition comprising the starch according to the third aspect of the invention and a food ingredient or water. This
- 35 aspect includes foods and non-food compositions and blends of the starch with other starches or starch-containing products.

In a fifth aspect the invention provides a composition comprising starch granules of the fourth aspect of the invention above and another food ingredient or water.

5 In a sixth aspect the invention provides a wheat plant which may be used to produce the grain or the starch granules or the starch of the previous aspects. The wheat plant may be transgenic or non-transgenic, as may be the grain it produces.

10 In a seventh aspect the invention provides a method of producing a wheat plant capable of producing grain comprising the steps of i) introducing a genetic variation into a parent wheat plant or seed; and ii) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to a wild-type plant or seed; wherein the grain comprises starch, wherein the proportion of amylose in the starch of the grain is at least 50%.

15 In a second form of the seventh aspect the invention provides a method of producing a wheat plant capable of producing grain comprising the steps of i) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a mutation of an SBEIIa gene or an introduced nucleic acid which encodes an inhibitor of SBEIIa gene expression, and ii) identifying a progeny plant or seed of the parent wheat
20 plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain; wherein the grain comprises starch and wherein the proportion of amylose in the starch of the grain is at least 30%. The step of introducing the genetic variation may comprise introducing an exogenous nucleic acid expressing an inhibitor of SBEIIa gene expression or may comprise
25 mutagenesis of the parent wheat plant.

In a third form of the seventh aspect the invention provides a method of producing a wheat plant capable of producing grain comprising the steps of i) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a
30 mutation of an SBEIIa gene, and ii) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain; iii) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a mutation of an SBEIIb gene, and iv) identifying a progeny plant or seed of
35 the parent wheat plant or seed which has a reduced level of SBEIIb gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain; v) crossing a plant having a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the

endosperm or both, with a plant having a reduced level of SBEIIb gene expression, SBEIIb enzyme activity in the endosperm or both; and identifying a wheat plant having reduced gene expression, enzyme activity or both, of both SBEIIa and SBEIIb.

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In a fourth form of the seventh aspect the invention provides a method of producing a wheat plant having a relative amylose content in starch of its grain of at least 50%, preferably having reduced activity of SBEIIa enzyme activity in the endosperm, which method comprises: a) identifying a wheat plant or grain having reduced SBEIIa activity expressed from the A, B or D genome of wheat; and b) crossing said wheat plant or a wheat plant produced from the grain of step a) with a second wheat plant having reduced SBEIIa activity; or c) crossing a plant having reduced SBEIIa enzyme activity with a plant having reduced SBEIIb enzyme activity; and identifying a wheat plant having reduced activity of both SBEIIa and SBEIIb. Preferably the plant of the seventh aspect is *Triticum aestivum* ssp. *aestivum*.

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In an eighth aspect, the invention provides a method of making altered starch, comprising altering a plant by the methods defined above and extracting the starch having altered properties.

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In a ninth aspect the invention provides a method of identifying a wheat plant or seed for a mutation in an SBEIIa gene, or an SBEIIb gene, comprising the steps of screening a population of wheat plants or seed with a molecular marker that is linked to the SBEIIb gene, or SBEIIa gene, respectively, of wheat; and identifying the plant or seed on the basis of the presence or absence of the linked molecular marker.

25

In a second form of the ninth aspect, the invention provides a method of identifying a wheat plant or seed for a mutation in an SBEIIa gene, or an SBEIIb gene, comprising the steps of screening a population of wheat plants or seed with an antibody that is specific for SBEIIb protein, or SBEIIa protein, respectively, of wheat; and identifying the plant or seed on the basis of the presence or absence of antibody binding.

30

In a tenth aspect, the invention provides grain obtained from a wheat plant, comprising a mutation wherein the SBEIIa gene is absent from the long arm of chromosome 2A or wherein the SBEIIa gene on the long arm of chromosome 2A comprises a mutation which leads to reduced SBEIIa protein, SBEIIa enzyme activity, or both, in the endosperm of said grain relative to wild-type grain. The mutation may be a null

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mutation of the SBEIIa gene or may be a deletion of at least part of the SBEIIa gene. The grain may further comprise a mutation wherein the SBEIIb gene is absent from the long arm of chromosome 2A or wherein the SBEIIb gene on the long arm of chromosome 2A comprises a mutation which leads to reduced SBEIIb protein, SBEIIb enzyme activity, or both, in the endosperm of said grain relative to wild-type grain. The deletion may disrupt expression of both the SBEIIa and SBEIIb genes on the long arm of chromosome 2A.

The plant may be a durum wheat plant that may further comprise a genetic variation which leads to reduced starch branching enzyme activity encoded by the SBEIIa gene on the long arm of chromosome 2B relative to wild-type grain. The further genetic variation may comprise an absence of the SBEIIa gene from the long arm of chromosome 2B or a mutation of the SBEIIa gene of the long arm of chromosome 2B which leads to reduced SBEIIa enzyme activity in the endosperm of said grain relative to wild-type grain.

The plant may be *Triticum aestivum* ssp *aestivum*, that perhaps additionally comprises a genetic variation which leads to reduced starch branching enzyme activity encoded by the SBEIIa gene on the long arm(s) of chromosome 2B, chromosome 2D or both chromosomes relative to wild-type grain. The further genetic variation comprises an absence of the SBEIIa gene from at least one of said chromosomes or a mutation of the SBEIIa gene of at least one of said chromosomes which leads to reduced SBEIIa enzyme activity in the endosperm of said grain relative to wild-type grain.

The plant may have introduced nucleic acid which encodes an inhibitor of SBEIIa gene expression, activity or both. The level of SBEIIa enzyme activity may be reduced by at least 40% relative to wild-type grain. The proportion of amylose in the starch of the grain may be at least 30% or at least 50%. The grain may be non-shrunken and may have an average weight of at least about 36 mg. At least 50% of starch granules from the grain may appear non-birefringent when observed under polarized light. The starch content of the grain when naked, in one form of the invention, is at least 25% (w/w) or has a starch content that is at least 90% of the starch content of wild-type grain.

The amylopectin of the grain of any of the forms of the present invention may have a reduced proportion of the 4 - 12 dp chain length fraction relative to the amylopectin of wild-type grain, as measured after isoamylase debranching of the amylopectin.

The grain may further comprising a reduced level of SBEI protein, SBEI enzyme activity or both and may further comprise an altered level of an enzyme relative to wild-type grain, wherein said enzyme is selected from the group consisting of ADP glucose pyrophosphorylase, GBSS, SSI, SSII, SSIII, a debranching enzyme of an isoamylase type, a debranching enzyme of a pullulanase type and any combination of these.

Forms of this tenth aspect of the invention encompass grain, starch granules extracted from the grain, and a product produced from the grain or its starch, such as, for example, flour, wholemeal, or semolina.

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BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. Sequence of the Starch Branching Enzyme IIa gene (*wSBE II-D1*) [SEQ ID No. 1] from *A. tauschii*, corresponding to the D genome *SBEIIa* gene of hexaploid wheat (*T. aestivum*).
- Figure 2. Partial wheat *SBEIIb* gene sequence (*wbe2b* genomic) [SEQ ID No. 2] from *T. aestivum*.
- Figure 3. Schematic of duplex-RNA constructs. A. The order of the gene elements used were promoter, *SBEIIa* or *SBEIIb* gene sequence (exons 1, 2 and 3) in sense orientation, intron (intron 3), *SBEIIa* or *SBEIIb* gene sequence (exons 1, 2, 3 and 4) in antisense orientation, and transcription terminator/polyadenylation sequence. B. The transcript of the ds-*SBEIIa* and ds-*SBEIIb* genes forms a "hairpin" RNA structure with a double-stranded region formed by hybridization between the sense and antisense sequences. The intron sequence bordered by the GT and AG nucleotides is spliced out.
- Figure 4. Starch granules observed through a light microscope from A) a wheat seed with wild type starch granules from the ds-*SBEIIa* transgenic line 83.1b, B) a wheat seed with distorted starch granules from the ds-*SBEIIa* transgenic line 50.1b.
- Figure 5. Birefringence of starch granules from wheat seed as for Figure 4, visualized under polarized light.

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- Figure 6. Comparison of partial wheat *SBEIIa* cDNA sequences. *sbe9* corresponds to part of AF338432.1 Partial sequences of the following are shown Y11282 [SEQ ID No. 3], sr997 [SEQ ID No. 4], sr995 [SEQ ID No. 5], *sbe9* [SEQ ID No. 6].
- Figure 7. PILEUP comparison of partial wheat *SBEIIa* sequences based on the first 63 amino acids. The probable genome location of the genes corresponding to the clones is indicated.
- Figure 8. Comparison of the deduced amino acid sequences of the D-genome polypeptide (sr854) [SEQ ID No. 7] with the product from the A or B genome (y11282) [SEQ ID No. 8]. The transit sequence (positions 1-54) is italicized.
- Figure 9. PCR amplification of an intron 3 region of *SBEIIb* gene from various wheat accessions (Lanes 1 to 11) using the primers ARA19F and ARA23R followed by digestion with *RsaI*. Bands corresponding to the A, B and D genomes are arrowed. Lane 3 (Aus17340) and Lane 5 (Aus10103) lack the D genome specific marker, while lane 8 (Aus12509) and lane 9 (Aus12565) lack the B genome marker.
- Figure 10. Southern hybridization of *HindIII* digested DNA from wheat accessions using a probe from the intron 3 region of *SBEIIb*. Lanes correspond to: 1) Aus12565, 2) Aus12509, 3) Aus10103, 4) CSDT2DL-4, 5) Aus12530 (durum wheat), 6) CSDT2BL-9, 7) Aus6323 8) CSDT2DS, 9) Aus17340, 10) Aus12745, 11) CSDT2DL-4 12) *Aegilops tauschii*.
- Figure 11. Screening of F2 population of the cross Aus17340a X Aus12509 by PCR amplification of the intron 3 region of *SBEIIb* using the primers AR2b19cF and AR2b23cR followed by *RsaI* digestion. Lane 8 lacks both the B and D genome markers, so line BD54 represents a BD double null line.

- Figure 12. Southern hybridization of *Hind*III (lanes 1 to 4) and *Eco*R1 (lane 5 to 8) digested BAC clones using a probe from the intron 3 region of *SBEIIb*. Lanes correspond to: 1) BAC 4, 2) BAC 5, 3) BAC 9, 4) BAC 12, 5) BAC 4, 6) BAC 5, 7) BAC 9, 8) BAC 12.
- Figure 13. A) FISH using a wSBEII-DA1 probe and a repetitive DNA sequence probe (pSc 119.2) to *A. tauschii* chromosomes (main photograph and lower insert) and wheat chromosomes (upper insert). B) FISH of SBEIIb probe to wheat chromosomes.
- Figure 14. SDS-PAGE analysis of granule bound proteins in wild-type Chinese Spring (CS) and SGP-1 null wheat lines at several stages of seed development (10, 15, 25 days after anthesis, M= mature) as indicated. The protein band intensity of the image of the silver stained gel was measured. The band intensity of GBSS in mature CS seed was normalized to 100 and the amount of other enzymes at indicated developmental stage is expressed as percentage of GBSS in mature CS. a) GBSS, b) SSI, c) SBEII. Black bars refer to SGP-1 null. An exemplary gel electrophoretogram for granule bound proteins from CS and the SGP-1 null line is shown.
- Figure 15. Relative amounts of SBEIIa and SBEIIb in the soluble fraction. Immunoblots of the SDS-PAGE were scanned and the protein band intensity of the images were measured. The amounts of proteins were estimated from SBEIIa- and SBEIIb-fusion proteins used on the gels as standard.
- Figure 16. A. Anion-exchange chromatography of wheat (cv Rosella) endosperm branching enzyme activities. Endosperm-soluble proteins were fractionated with ammonium sulphate and chromatographed on a Sephacryl S-200 column prior to application to a Resource Q anion exchange column. B) Immuno-detection analysis using anti-WBE1 antibody of wheat endosperm SBEI separated on a non-denaturing PAGE. The SBEI protein bands labelled as A and B are products from the A and B genomes respectively and Di and Dii are products from the D genome. Lanes

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correspond to extracts from: 1. CS, lane 2. N7BT7A, lane 3. N7AT7B, lane 4. N7DT7A. C) Immuno-detection analysis of purified fractions representing active peaks in the anion-exchange chromatogram using anti-WBE 1 antibody. Lane 1. endosperm crude soluble extract, lane 2. fractions representing peak 1, lane 3. fraction representing peak 2.

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Figure 17. Screening of doubled haploid progeny of the cross VC3.1.11 x CS7AL-15 for segregation of SBEI isoforms by immuno-detection using anti-WBE I antibody. Lanes 1 to 14 correspond to doubled haploid progeny lines. Lane 6 is a triple null SBEI mutant line designated as A113 and lane 7 is a line normal for SBEI isoforms designated as D28.

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Figure 18. PCR amplification of DNA from gamma ray induced mutant seeds (Lanes 1 to 6) of the cross Veery 3 x Gabo 1BL.1RS using the primers AR2b19cF/AR2b23cR. Lane 2 represents the mutant seed MLT2B8 and Lane 7 represents Chinese Spring.

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Figure 19. PCR amplification of DNA from wheat lines using A genome specific primers for wheat SBEIIa gene, ARIIaAF/ARIIaAR. Lanes in the order of 1 to 5 are CS, MLT2B8, MLT2D1, Dt2AS and BD219 (plant that is null mutant for both *SBEIIa* and *SBEIIb* plant in both B and D genomes).

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Figure 20. Sepharose CL 2B gel chromatogram of starch from wheat lines a) Acc144008 and b) Acc 144087, assayed using starch assay kit (Sigma).

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Figure 21. Chain length profile comparison of starches from wheat transgenic lines with respect to that of non transformed control, NB1 (wheat). The percentage of total mass of individual oligosaccharides from starches from the non transformed control is subtracted from the corresponding values from starches from transgenic lines. Samples are 085 (♦), 025 (▲), 008 (O).

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DETAILED DESCRIPTION OF THE INVENTION

Alteration of SBEIIa in wheat

5 The invention is based on the finding that a reduction in SBEIIa activity in wheat endosperm results in modified starch production, particularly high relative amylose levels in the wheat grain. This unexpected result is in contrast to the findings in maize and rice where mutation in SBEIIa did not alter the amylopectin/amylose profile (Blauth et al., 2001, Nakamura, 2002). In a further embodiment, there is an alteration in one or
10 more additional starch biosynthetic enzyme activities, such as a reduction in SBEIIb as well as SBEIIa activity. Mutation in the genes encoding these two activities is aided by the surprising finding that *SBEIIa* and *SBEIIb* are closely linked in wheat, in contrast to non-linkage in maize and rice. We have also found, unexpectedly, that grain of the wheat plant which has reduced levels of SBEIIa and SBEIIb activity is non-shrunken.

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Method of producing a wheat plant

In one aspect, the invention provides a method of producing a wheat plant having altered starch in its grain, in particular of increasing the proportion of amylose in the starch to at least 30%. Ordinarily in hexaploid and durum wheats, the proportion of amylose in
20 starch ranges from about 18 to about 30%, in certain mutants (SGP-1 deficient) up to about 35%. In one embodiment, the method of the invention comprises the step of introducing a genetic variation into a parent wheat plant or seed, to provide wheat plants that produce grain having starch comprising at least 30% amylose. The proportion of amylose in the starch as defined herein is on a weight/weight (w/w) basis, i.e. the weight
25 of amylose as a percentage of the weight of starch from the grain. In further embodiments, the proportion of amylose in the starch is at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70% or at least 75% (each w/w). In further embodiments of the invention, the method provides for a proportion of amylose of at least 80% or at least 90% (w/w).

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In a further embodiment, the method includes altering, preferably reducing, the level of starch branching enzyme IIa (SBEIIa) protein, enzyme activity or both in the endosperm of wheat. That is, a genetic variation that is introduced into the wheat plant leads, directly or indirectly, to the change in the level of SBEIIa and consequently to the starch
35 modifications described herein. In a further embodiment which is not mutually exclusive with the previous embodiment, the method comprises the alteration, preferably reduction, of the level of expression of the *SBEIIa* gene in the endosperm of wheat, or it comprises

the mutation of an *SBEIIa* gene in wheat, whereby the *SBEIIa* activity in endosperm is reduced. A reduction in the level of *SBEIIa* gene expression or of other genes may be achieved by the introduction of a nucleic acid, for example a transgene, which encodes an inhibitory molecule. Examples of inhibitory molecules include antisense, co-suppression, ribozyme or duplex RNA molecules.

As used herein, the terms “altering”, “increasing”, “increased”, “reducing”, “reduced”, “inhibited” or the like are considered relative terms, i.e. in comparison with the wild-type or unaltered state. The “level of a protein” refers to the amount of a particular protein, for example *SBEIIa*, which may be measured by any means known in the art such as, for example, Western blot analysis or other immunological means. The “level of an enzyme activity” refers to the amount of a particular enzyme measured in an enzyme assay. It would be appreciated that the level of activity of an enzyme might be altered in a mutant but not the expression level (amount) of the protein itself. Conversely, the amount of protein might be altered but the activity remain the same if a more or less active protein is produced. Reductions in both amount and activity are also possible such as, for example, when a gene encoding the enzyme is inactivated. In certain embodiments, the reduction in the level of protein or activity is by at least 40% or by at least 60% compared to the level of protein or activity in the endosperm of unmodified wheat, or by at least 75%, at least 90% or at least 95%. The reduction in the level of the protein or enzyme activity or gene expression may occur at any stage in the development of the grain, particularly during the grain filling stage while starch is being synthesized in the developing endosperm, or at all stages of grain development through to maturity.

“Starch” is defined herein as polysaccharide made up essentially of α -glucopyranose units. Starch is the major storage carbohydrate in wheat, is synthesized in the amyloplasts and formed and stored in granules. It includes amylose, an essentially linear α -1,4-D-glucopyranose polymer and amylopectin, which has short chains of α -D-glucopyranose units primarily linked by α -1,4 bonds with α -1,6 linked branches. Wheat starch from wild-type plants comprises up to about 20%-30% of amylose and about 70%-80% of amylopectin. A further significant difference between the two molecules is their molecular weight. Amylose has a helical conformation with a molecular weight of 10^4 - 10^6 while amylopectin has a molecular weight of about 10^7 to 10^8 daltons. Recent studies have shown up to about 0.1% of α -1,6-glycosidic branching sites may occur in amylose, therefore it is described as “essentially linear”. “Amylose” is defined herein as including essentially linear molecules composed of α -1,4 linked glucosidic (glucopyranose) units and amylose-like long-chain amylopectin (sometimes referred to

- as "intermediate material" or "amylose-like amylopectin", Takeda et al., 1993b; Fergason, 1994). Amylose content may be determined by any of the methods known in the art including size exclusion HPLC, for example in 90% (w/v) DMSO, concanavalin A methods (Megazyme Int, Ireland), or preferably by iodometric methods, for example as described in Example 1. The HPLC method may involve debranching of the starch (Batey and Curtin, 1996) or not involve debranching. From the grain weight and amylose content, the amount of amylose deposited per grain can be calculated and compared for transgenic and control lines.
- 10 In another embodiment, the method comprises the step of determining the amount or the activity of SBEIIa in wheat endosperm using any method known in the art. In a certain embodiment, the level of the protein is measured, for example by immunodetection methods such as Western blotting or ELISA assays, or the level of its corresponding mRNA is measured by methods well known in the art such as Northern blot
- 15 hybridization analysis or reverse transcription polymerase chain reaction (RT-PCR). In another embodiment, the method comprises the step of selecting or screening for a wheat plant or grain having an altered level of SBEIIa protein or enzyme activity in its endosperm. The selection step may be based on a reduced level of the SBEIIa activity or protein, or it may be based on the phenotype of the grain of the wheat plant such as an
- 20 increased proportion of amylose or decreased proportion of amylopectin, or a visual phenotype, for example shrunken grain or altered starch granule properties.
- It would be appreciated that the invention includes a method of identifying a wheat plant with the altered starch properties in its grain using any of the methods as described
- 25 herein, either directly determining the starch properties or indirectly, for example, detecting the presence of a genetic variation in the plant or its grain. The plant may be a plant in a population of wheat plants, such as, for example, in wheat breeding.
- SBE activity may be measured directly by enzyme assay, for example by the
- 30 phosphorylase stimulation assay (Boyer and Preiss, 1978). This assay measures the stimulation by SBE of the incorporation of glucose 1-phosphate into methanol-insoluble polymer (α -D-glucan) by phosphorylase a. SBE activity can be measured by the iodine stain assay, which measures the decrease in the absorbance of a glucan-polyiodine complex resulting from branching of glucan polymers. SBE activity can also be assayed
- 35 by the branch linkage assay which measures the generation of reducing ends from reduced amylose as substrate, following isoamylase digestion (Takeda et al., 1993a). Preferably, the activity is measured in the absence of SBEI or SBEIIb activity. Isoforms

- of SBE show different substrate specificities, for example SBEI exhibits higher activity in branching amylose, while SBEIIa and SBEIIb show higher rates of branching with an amylopectin substrate. The isoforms may also be distinguished on the basis of the length of the glucan chain that is transferred. SBE protein may also be measured by using specific antibodies such as those described herein. The SBEII activity may be measured during grain development in the developing endosperm, or alternatively in the mature grain where the protein is still present in equivalent, but unaltered, grain and can be assayed by immunological methods.
- In a further aspect, the invention provides a method of altering, preferably reducing, the activity of multiple starch biosynthesis enzymes in wheat endosperm, wherein one of the enzymes is SBEIIa, such that the proportion of amylose in the starch of the grain is at least 50%. In certain embodiments, the levels of both SBEIIa and SBEIIb proteins or enzyme activities are reduced or the levels of all three of SBEIIa, SBEIIb and SBEI are reduced. Other starch biosynthesis enzymes that may be altered in combination with SBEIIa are: SSI, SSII, SSIII. Starch debranching enzymes may also be altered, for example the activity of isoamylase or pullulanase. Any combination of the above enzymes is also provided, so long as SBEIIa is altered. In a further embodiment, the activity of one or more starch biosynthesis enzyme is altered in the plant in tissues other than endosperm, for example the activity of SBEI or SBEII may be increased in leaves to compensate for some loss of activity caused by a transgene encoding an SBEIIa-inhibitory molecule intended primarily for expression in the endosperm. The alteration may be an increase or reduction in amount or an alteration in the timing of expression, for example. Alternatively, starch synthesis may be further improved by the overexpression of one or more starch biosynthetic enzymes in combination with a reduction in SBEIIa. Genes encoding such enzymes may be from any of a variety of sources, for example from bacterial or other sources other than wheat, and may be modified to alter the catalytic properties, for example alteration of the temperature dependence of the enzymes (for example, see WO94/09144).
- The high amylose phenotype may be achieved by partial or full inhibition of the expression of the *SBEIIa* gene, or the *SBEIIa* and *SBEIIb* genes. The extent to which the gene or genes are inhibited will in some degree determine the characteristics of the starch made in the wheat grain. Any of a range of gel electrophoresis techniques carried out on the proteins extracted from the modified wheat endosperm will reveal the nature and extent of modification to the SBEIIa and/or SBEIIb activity. Modification may occur as a reduction in SBEIIa and/or SBEIIb activity, complete abolition of enzyme

activity, or an alteration in the distribution of the SBEIIb or other enzymes within the endosperm. To carry out these tests, starch may be extracted from the wheat endosperm and the proteins therein analyzed, for example as outlined in Rahman et al, 1995.

Techniques well known in the art such as SDS-PAGE and immunoblotting are carried out on the soluble and the starch granule fractions and the results used to identify the plants or grain where modifications have occurred to the SBEIIa and/or SBEIIb enzymes.

Wheat plants

In a further aspect, the invention provides a wheat plant capable of producing grain having a proportion of amylose in the starch of at least 30%. In further embodiments, the proportion of amylose is at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70% or at least 80%. In another embodiment, the wheat plant whose grain comprises any of these levels of amylose in its starch, comprises a genetic variation which leads to a reduction in the level of *SBEIIa* gene expression, SBEIIa enzyme activity in the endosperm, or both, relative to wild-type grain. In a preferred embodiment, the genetic variation comprises a mutation of an *SBEIIa* gene or an introduced nucleic acid which encodes an inhibitor of *SBEIIa* gene expression. The inhibitor may comprise an antisense, co-suppression, ribozyme, or duplex RNA or similar molecule that inhibits *SBEIIa* expression and/or activity.

A wheat plant is defined herein as any plant of a species of the genus *Triticum*, which species is commercially cultivated, including, for example, *Triticum aestivum* L. ssp. *aestivum* (common or bread wheat), other subspecies of *Triticum aestivum*, *Triticum turgidum* L. ssp. *durum* (durum wheat, also known as macaroni or hard wheat), *Triticum monococcum* L. ssp. *monococcum* (cultivated einkorn or small spelt), *Triticum timopheevi* ssp. *timopheevi*, *Triticum turgidum* L. ssp. *dicoccon* (cultivated emmer), and other subspecies of *Triticum turgidum* (Feldman). The wheat may be hexaploid wheat having an AABBDD type genome, or tetraploid wheat having an AABB type genome. Since genetic variation in wheat according to the invention can be transferred to certain related species including rye and barley by hybridization, the invention also includes the hybrid species thus formed, including triticale that is a hybrid between bread wheat and rye. In a particular embodiment, the wheat plant is of the species *Triticum aestivum*, and preferably of the subspecies *aestivum*. Alternatively, since mutations or transgenes can be readily transferred from *Triticum aestivum* to durum wheat, the wheat is preferably *Triticum turgidum* L. ssp. *durum*.

The invention also provides wheat plants with a reduced level of *SBEIIa* protein, enzyme activity in the endosperm, or both, the wheat plant being capable of producing grain having starch comprising an increased proportion of amylose compared to starch extracted from wild-type plants. The reduced level of *SBEIIa* may occur during at least
5 part of the development process of the grain, or throughout the process to maturity. In a further embodiment, the level of *SBEIIa* is reduced in the endosperm by at least 50%, at least 75%, at least 90% or at least 95% compared to the wild-type. The term "wild-type" has its normal meaning in the field of genetics and includes wheat cultivars or genotypes which are not modified as taught herein.

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The invention also provides progeny plants and grain which have the desired characteristics of the parent wheat plants, in genotype and/or phenotype. The invention also extends to any propagating material of the wheat plants that can be used to produce the plants with the desired characteristics, such as cultured tissue or cells.

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The invention also encompasses wheat plants that have altered, preferably reduced, *SBEIIb* or other starch biosynthetic enzymes in addition to reduced *SBEIIa* activity. Plants having reduced *SBEIIa* and *SBEIIb* activities may be produced by crossing a plant reduced for *SBEIIa* with a plant reduced for *SBEIIb*, or by introducing a transgene
20 encoding a molecule that inhibits expression of both *SBEIIa* and *SBEIIb* genes. Because of the close linkage of the *SBEIIa* and *SBEIIb* genes in wheat as revealed herein, plants reduced for both activities may also be produced by identifying varieties lacking the *SBEIIa* and *SBEIIb* isoforms encoded by one of the genomes of wheat, and crossing such varieties to produce a plant reduced for the isoforms encoded by at least
25 two genomes.

The invention also encompasses the genetic variations(s) or mutations in other genetic backgrounds or other species which can be hybridised with the wheat plant as described above. The altered (mutant) plants may be crossed with plants containing a more
30 desirable genetic background. After the initial crossing, a suitable number of backcrosses may be carried out to remove the less desirable background. The desired genetic background may include a suitable combination of genes providing commercial yield and other characteristics such as agronomic performance or abiotic stress resistance. The genetic background might also include other altered starch biosynthesis
35 or modification genes, for example genes from other wheat lines that have a shrunken endosperm where the causal gene is not known.

The plants may be transgenic or non-transgenic.

The invention also provides wheat plants comprising a mutation wherein the *SBEIIa* gene is absent from the long arm of chromosome 2A (2AL) or wherein the *SBEIIa* gene on the long arm of chromosome 2A comprises a mutation which leads to reduced level of *SBEIIa* enzyme activity in the endosperm of said grain relative to wild-type grain. Despite an extensive screen of 2400 wheat accessions, the inventors did not find such plants that were naturally occurring, suggesting that selection for retention of the functional *SBEIIa* gene on 2AL might be happening in nature. However, such plants could be produced and identified after mutagenesis. These plants are non-transgenic which is desirable in some markets. These plants may be bread wheat, durum wheat or other wheat. In a preferred embodiment, the wheat plant comprises a deletion of at least part of the *SBEIIa* gene, which may extend to at least part of the *SBEIIb* gene, on the 2AL chromosome. As is understood in the art, hexaploid wheats such as bread wheat comprise three genomes which are commonly designated the A, B and D genomes, while tetraploid wheats such as durum wheat comprise two genomes commonly designated the A and B genomes. Each genome comprises 7 pairs of chromosomes which may be observed by cytological methods during meiosis. The chromosomes are commonly designated in order according to their size from largest to smallest, chromosome 2 therefore being the second largest chromosome in each genome. Each chromosome has a centromere, which on chromosome 2 is positioned asymmetrically; therefore the two arms of chromosome 2 are designated "short" and "long". The "long arm of chromosome 2A" is defined herein as the region of that chromosome between the centromere and tip along the long arm, in accord with the standard meaning of the term. The terms "long arm of chromosome 2B" and the "long arm of chromosome 2D" are defined in the same way except that they relate to chromosome 2 of the B or D genomes of wheat, respectively.

We have found that the *SBEIIa* and *SBEIIb* genes are closely linked on chromosome 2 in wheat. In a particular embodiment, the wheat plant comprises the majority (>50%) of 2AL, which chromosome arm comprises a mutation of at least the *SBEIIa* gene. That is, chromosome 2AL is essentially present, comprising a mutation in at least the *SBEIIa* gene of the A genome. The presence of 2AL may be determined by cytological techniques such as, for example, in situ hybridization techniques (see Example 9) or by using 2AL specific molecular markers. In a preferred embodiment, the wheat plant is homozygous for said mutation. The mutation may be a null mutation. The mutation may be a deletion.

In a particular embodiment, the deletion allele is derived from the MLT2B8 or MLT2D1 plants. As the mutant *SBEIIa* alleles in these plants occur on the 2AL chromosome, these alleles can be introduced into varieties of bread wheat or durum wheat by crossing, and
5 the invention therefore includes such plants, and grain and starch products obtained therefrom. These alleles may be combined with other useful starch biosynthesis genes or alleles, or other useful genetic traits.

The invention clearly extends to methods of producing or identifying such wheat plants
10 or the grain produced by such plants.

Grain

The invention also provides wheat grain comprising an altered starch compared to starch extracted from wild-type wheat grain. Grain is defined herein as essentially mature
15 grain. This includes grain as harvested in a commercial setting. In one embodiment, the altered starch is at least partly a consequence of reduced *SBEIIa* activity during development of the endosperm of the wheat grain. In a further embodiment, which is not mutually exclusive with the previous embodiment, the grain comprises an increased proportion of amylose (as a percentage of total starch). This may be determined as a
20 reduced proportion of amylopectin in the starch compared to grain from a wild-type plant. Wild-type wheat starch has approximately 20-30% amylose and 70-80% amylopectin. The grain of the invention comprises starch preferably comprising at least 50% (w/w) amylose. In a further embodiment, both *SBEIIa* and *SBEIIb* activities are reduced during development of the endosperm. In a further embodiment, the activity of
25 *SBEI* is also reduced. In further embodiments, the proportion of amylose, as measured by methods well understood in the art, is at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80% or at least 90% (each w/w) of the starch of the grain. Increased amylose levels may be evidenced by abnormal starch granule morphology or loss of birefringence of the granules when observed under a light microscope or other
30 methods known in the art. In a particular embodiment, the proportion of amylose is measured by an iodometric method, which may be a spectrophotometric method such as, for example, the method of Morrison and Laignelet (1983), or by high-performance liquid chromatography (HPLC, for example, Batey and Curtin, 1996).

35 In further embodiments, the wheat grain comprises starch that has altered physical characteristics such as, for example, an increased or reduced gelatinisation temperature, altered swelling characteristics during or following gelatinisation, altered viscosity, an

altered chain length distribution in the amylopectin, or any combination of these. The increased or reduced gelatinisation temperature may be for the first peak of gelatinisation, the second peak, or both. One or more properties of the starch such as, for example, the enthalpy of gelatinisation, may be unaltered. The temperature of the first peak (apex) of gelatinisation as measured by differential scanning calorimetry may be increased by at least 3°C or 5°C, preferably by at least 7°C or 8°C and more preferably by at least 10°C compared to the temperature of the first peak for the corresponding starch from wild-type grain. In a particular embodiment, the increase is in the range of 3°C to 12°C.

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The grain may be shrunken or non-shrunken, preferably having a non-shrunken phenotype. "Non-shrunken" as used herein is defined as where the majority of grains, preferably at least 90% of the individual grains, show a plump or fully-filled phenotype. This is usually associated with a normal or near normal level of starch accumulation. In contrast, a "shrunken" phenotype as used herein refers to the majority of grains, particularly at least 90% of the grains, having reduced starch accumulation. Slightly shrunken grain refers to a reduction in average starch content of at least 30%, moderately shrunken grain refers to a reduction in average starch content of at least 50%, and highly shrunken grain refers to a reduction in average starch content of at least 70%, each relative to wild-type grain. Shrunkenness may also be measured by the relative starch content, as a percentage of mature grain weight. Unaltered field-grown wheat grain has a starch content of about 65%, while in shrunken grain this is reduced to less than 50%.

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In further embodiments, the grain has an average weight of at least 36mg or 40mg. The average weight of the grain is determined by measuring the weight of a known number of grains, being a representative sample of the batch of grain, and dividing the total weight by the number of grains. It would be appreciated that characteristics of the grain such as starch content, average weight and a non-shrunken phenotype that are near wild-type levels are desirable for commercial production of the grain.

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The invention also provides flour, meal, dough or other products produced from the grain or using the grain. These may be unprocessed or processed, for example by fractionation or bleaching. The invention further provides wheat grain useful for food production obtained from the wheat plant of the invention. Additionally the invention encompasses grain that has been processed in other ways, so that the grain may have been milled, ground, rolled, pearled, kibbled or cracked, or par boiled (polenta), for example as cous cous.

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Starch

In another aspect, the invention provides starch obtained from the grain of the wheat plants as described herein, the starch having an increased proportion of amylose and a reduced proportion of amylopectin. In a preferred embodiment, the starch is obtained from grain of a wheat plant which has a reduced level of SBEIIa protein, SBEIIa enzyme activity in the endosperm, or both, relative to wild-type wheat. In a further embodiment, both SBEIIa and SBEIIb activities are reduced, or all three of SBEIIa, SBEIIb and SBEI are reduced relative to wild-type wheat.

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In a further aspect, the invention provides starch obtained from the grain of the wheat plants as described herein, comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80% or at least 90% amylose. The starch is at least partly purified, i.e. it has been separated from at least one other component of the grain. Purified starch may be obtained from grain by a milling process, for example a wet milling process, which involves the separation of the starch from protein, oil and fibre. The initial product of the milling process is a mixture or composition of starch granules, and the invention therefore encompasses such granules, comprising the modified starch as described herein.

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The starch may have an increased or reduced gelatinisation temperature, preferably an increased gelatinisation temperature. In particular embodiments, at least one of the temperature of onset of the first peak or the temperature for the apex of the first peak is increased by at least 3°C, at least 5°C, at least 7°C or at least 10°C as measured by DSC compared to starch extracted from wild-type wheat grain. In a particular embodiment, the increase is in the range of 3°C to 12°C. Of particular note, the gelatinisation temperature may have a decreased temperature of onset of the first peak combined with an increased temperature of the peak apex. In another embodiment which is not mutually exclusive with the previous, the starch has an altered gelatinisation temperature for the first peak but exhibits a substantially unaltered temperature for the second peak, which corresponds to amylose-lipid dissociation, as determined by DSC. In a further embodiment, the starch exhibits a decreased enthalpy during gelatinisation, such as, for example, a decrease by at least 25% or at least 40% compared to that of corresponding wild-type wheat starch.

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In another embodiment, the starch comprises an elevated level of resistant starch, with an altered structure indicated by specific physical characteristics. Such characteristics may

include physical inaccessibility to digestive enzymes which may be by reason of having altered starch granule morphology, the presence of appreciable starch associated lipid, altered crystallinity, altered amylopectin chain length distribution, or any combination of these. The high proportion of amylose also contributes to the level of resistant starch.

5

The invention also provides starch from grain of the exemplified wheat plant comprising increased amounts of dietary fibre, preferably in combination with an elevated level of resistant starch. This increase is also at least in part a result of the high relative level of amylose.

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The invention clearly extends to methods of producing the wheat starch described herein. In one embodiment, the method comprises the steps of obtaining wheat grain as described herein and extracting the starch from the grain. The wheat grain may be obtained by growing the wheat plants described herein and harvesting the grain, or from a producer of the grain or importer of the grain.

15

Methods of reducing gene activity

The expression and/or activity of SBEIIa, SBEIIb or other starch biosynthesis or modification genes may be altered by introducing one or more genetic variations into the wheat plant. As used herein, a "genetic variation" means any heritable alteration in the genome of the wheat plant which, in this context, affects the expression or activity of the gene of interest. Genetic variations include mutations such as point mutations, insertions, substitutions, inversions, duplications, translocations and preferably deletions, and the introduction of one or more transgenes into the genome.

25

The phrases "nucleic acid molecule" and "nucleic acid sequence" as used herein refer to a polymer of nucleotides, which may be single-stranded or double-stranded. It may comprise DNA such as, for example, genomic DNA or cDNA, or RNA, mRNA or any combinations of these. For introduction into wheat cells, a nucleic acid molecule may be chemically modified for improved delivery or stability, or protected as part of a vector such as a viral vector. The nucleic acid molecule may be obtained by cloning techniques or synthesized by techniques well known in the art. The nucleic acid molecule may comprise a coding strand or non-coding strand (antisense) or a combination of these such as, for example, in inverted repeat constructs. In reference to nucleic acid sequences which "correspond" to a gene, the term "correspond" refers to a nucleotide sequence relationship, such that the nucleotide sequence has a nucleotide sequence which is the same as the reference gene or an indicated portion thereof, or has a nucleotide sequence

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which is exactly complementary in normal Watson-Crick base pairing, or is an RNA equivalent of such a sequence, for example, an mRNA, or is a cDNA derived from an mRNA of the gene.

5 Nucleotide sequences are presented herein by a single strand sequence in the 5' to 3' direction, using the standard one letter nucleotide abbreviations. "Complementary" describes the relationship between two single-stranded nucleic acid molecules or sequences that anneal by base-pairing. For example, 5'-GACT-3' pairs with its complement, 5'-AGTC-3'. "Homology" or "homologous" refers to sequence similarity
10 or identity between two or more nucleotide sequences or two or more polypeptide sequences, according to the context. The term "percent identity" as applied to nucleotide sequences refers to the percentage of nucleotide matches between two nucleotide sequences aligned using a standardized algorithm such as, for example, the CLUSTAL V algorithm or the Blastn or BLAST 2 Sequences programs available from the National
15 Center for Biotechnology Information, available on the Internet at <http://www.ncbi.nlm.nih.gov/BLAST/>, and preferably set at default parameters. In similar fashion, "percent identity" may refer to polypeptide sequences.

Reference herein to a "gene" including an SBEIIa, SBEIIb or other starch biosynthetic
20 gene, or genes encoding antisense, co-suppression, ribozyme, duplex RNA molecules or the like, is to be taken in its broadest context and includes a classical genomic gene having a transcribed region associated with regulatory regions such as promoters and transcription terminators-polyadenylation sequences. The transcribed region includes transcribed but not translated sequences (untranslated sequences, UTR) and optionally
25 may include a protein coding region or introns, which are spliced out to form a mature RNA, or any combination of these. A "gene" includes forms obtained from cDNA, corresponding to the exons, and RNA genes such as those found on RNA genomes. The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product.

30 When present in a cell, preferably a wheat cell, a "gene" directs the "expression" of a "biologically active" molecule or "gene product", which may be RNA or a polypeptide. This process is most commonly by transcription to produce RNA and translation to produce protein. Such a product may be subsequently modified in the cell. RNA may be
35 modified by, for example, polyadenylation, splicing, capping, dicing into 21-23 nucleotide fragments, or export from the nucleus or by covalent or noncovalent interactions with proteins. Proteins may be modified by, for example, phosphorylation,

glycosylation or lipidation. All of these processes are encompassed by the term "expression of a gene" or the like as used herein.

As used herein, the terms "wheat *SBEIIa* gene" and "wheat *SBEIIb* gene" and related
5 terms refer to the genes that have been identified from wheat that encode *SBEIIa* or
SBEIIb enzymes, respectively, and homologous genes present in other wheat varieties.
These include, but are not limited to, the gene sequences listed in Table 1. It would be
understood that there is natural variation in the sequences of *SBEIIa* and *SBEIIb* genes
from different wheat varieties. The homologous genes are readily recognizable by the
10 skilled artisan. The degree of sequence identity between homologous *SBEIIa* genes or
the proteins is thought to be at least 90%, similarly for *SBEIIb* genes or proteins.

The genes for use in the invention may be derived from a naturally occurring *SBEIIa*,
SBEIIb or other starch biosynthetic gene by standard recombinant techniques. A
15 "recombinant nucleic acid molecule" or like term as used herein refers to a sequence that
is not naturally occurring or has a sequence that is made by an artificial combination of
two or more otherwise separated segments of sequence. This artificial combination may
be formed by chemical synthesis or, more commonly, by the artificial manipulation of
isolated segments of nucleic acids, for example by genetic engineering techniques well
20 known in the art. The term "recombinant" includes nucleic acids that have been altered
solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a
recombinant nucleic acid may include a nucleic acid sequence operably linked to a
promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used,
for example, to transform a cell.

25 Generally, a gene may be subjected to mutagenesis to produce single or multiple
nucleotide substitutions, deletions and/or additions such as, for example, codon
modification. Nucleotide insertional derivatives of such genes include 5' and 3' terminal
fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional
30 nucleotide sequence variants are those in which one or more nucleotides are introduced
into a predetermined site in the nucleotide sequence, although random insertion is also
possible with suitable screening of the resulting product. Deletional variants are
characterised by the removal of one or more nucleotides from the sequence.
Substitutional nucleotide variants are those in which at least one nucleotide in the
35 sequence has been removed and a different nucleotide inserted in its place. Such a
substitution may be "silent" in that the substitution does not change the amino acid
defined by the codon. Alternatively, conservative substituents are designed to alter one

amino acid for another similar acting amino acid. Typical substitutions are those made in accordance with the following:

Suitable residues for conservative amino acid substitutions

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
5	Ala	Ser
	Arg	Lys
	Asn	Gln; His
	Asp	Glu
10	Cys	Ser
	Gln	Asn
	Glu	Asp
	Gly	Ala
	His	Asn; Gln
15	Ile	Leu; Val
	Leu	Ile; Val
	Lys	Arg; Gln; Glu
	Met	Leu; Ile
	Phe	Met; Leu; Tyr
20	Ser	Thr
	Thr	Ser
	Trp	Tyr
	Tyr	Trp; Phe
	Val	Ile; Leu
25		

Transgenes

The expression and/or activity of *SBEIIa*, *SBEIIb* or other starch biosynthesis or modification genes may be altered by introducing one or more transgenes into the wheat
 30 plant. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology and which has been introduced into the organism or cell, preferably wheat cell, of interest. The transgene may include genetic sequences derived from the organism or cell, for example an antisense sequence. The transgene
 35 typically includes an exogenous nucleic acid which is not derived from said organism or cell. "Transgenic" refers to the organism or cell containing a transgene. "Non-transgenic" refers to the absence of any transgene in the genome. A transgene is preferably integrated into the genome of the organism or cell, for stable inheritance.

40 Those skilled in the art will be aware that expression of an gene, or a complementary sequence thereto, in a cell, requires said gene to be placed in operable connection with a promoter sequence. The choice of promoter for the present purpose may vary depending upon the level of expression required and/or the tissue, organ and species in which expression is to occur, particularly endosperm specific promoters.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, of the nucleic acid molecule it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include promoters derived from the genes of viruses, yeast, moulds, bacteria, insects, birds, mammals and plants, preferably those capable of functioning in plant cells, more preferably those capable of being expressed in the endosperm of wheat. The promoter may regulate expression constitutively, or differentially, with respect to the tissue in which expression occurs. Alternatively, expression may be differential with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or temperature.

The method of reducing *SBEIIa* or other starch biosynthetic gene activity may comprise the step of introducing a transgene into a regenerable cell of wheat and regenerating a transgenic wheat plant from the transformed cell. The branching enzymes involved in synthesis of amylopectin include *SBEI*, *SBEIIa* and *SBEIIb* and the invention encompasses a reduced expression of *SBEIIa* alone or in combination with alteration of *SBEIIb* or *SBEI* expression. Therefore, the transgene(s) may inactivate more than one of these genes. Moreover, the inactivation of *SBEIIb* and/or *SBEI* may be direct, in that the transgene (e.g. encoding duplex RNA, antisense, or ribozyme RNA, see below) directly targets the *SBEIIb* or *SBEI* gene expression, or it may indirectly result in the alteration in the expression of *SBEIIb* or *SBEI*. For example, the transgene RNA may target only the *SBEIIa* gene/RNA in terms of sequence identity or basepairing but also result in

reduction of *SBEIIb* or *SBEI* activity by altering protein stability or distribution in the endosperm. Additionally forms of the present invention reside in the combination of an altered activity of *SBEIIa* and an alteration of one or more other amylopectin synthesis enzymes, which enzymes may include SSI, SSII, SSIII, and debranching enzymes such as isoamylase or pullulanase. Expression of any or all of these may be altered by introduction of a transgene.

Several DNA sequences are known for amylopectin synthesis genes in wheat, any of which can be the basis for designing transgenes for inactivation of the genes in wheat. These include *SBEIIa* (GenBank accession numbers Y11282, AF338431 and AF338432) and *SBEIIb* (WO 00/15810, WO 01/62934). The *SBEI* gene of wheat is described in Rahman et al., (1997) and Rahman et al., (1999). The *Triticum tauschii* sequence for *SBEI*, which is highly homologous to the wheat D genome *SBEI* gene, can be found in published Patent specification WO 99/14314. A cDNA sequence for *SBEI* of wheat can be accessed in the GenBank database under accession number AF076679. Homologues of other amylopectin synthesising genes from barley or other closely related species can also be used to modify gene expression levels in wheat. Such genes or fragments thereof can be obtained by methods well known in the art, including PCR amplification or hybridization to labeled probes.

“Stringent hybridization conditions” as used herein means that hybridization will generally occur if there is at least 90% and preferably at least 95% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (1xSSC = 150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt’s solution, 10% dextran sulfate, and 20 µg/ml denatured sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

The region(s) of the homologues used in preparing the transgene construct should have at least 85% identity to the corresponding wheat gene, preferably at least 90% and even more preferably 95-100% identity in the appropriate region. It is also preferred that the transgene specifically target the amylopectin synthesis genes expressed in the endosperm of wheat and have less or minimal effect on amylopectin synthesis elsewhere

in the plant. This may be achieved by use of suitable regulatory sequences such as endosperm-specific promoters in the transgene.

Antisense

5 Genetic engineering approaches to altering, in particular specifically reducing, gene activity in plants such as wheat are well known in the art. These methods include the introduction of gene constructs for expression of a suitable antisense molecule that is complementary to the RNA of the target gene and can hybridize with it. Antisense
10 molecules are thought to interfere with the translation or processing or stability of the mRNA of the target gene, thereby inactivating expression of the gene. Methods of devising antisense sequences are well known in the art and examples of these can be found in United States Patent No. 5190131, European patent specification 0467349-A1, European patent specification 0223399-A1 and European patent specification 0240208, which are incorporated herein by reference. The use of antisense methods in plants has
15 been reviewed by Bourque (1995) and Senior (1998). Bourque lists a large number of examples of gene inactivation using antisense sequences in plant systems. She also states that attaining 100% inhibition of an enzyme activity may not be necessary as partial inhibition will more than likely result in measurable change in the system. Senior (1998) states that antisense methods are now a very well established technique for
20 manipulating gene expression in plants.

Antisense molecules for wheat *SBEIIa*, *SBEIIb*, *SBEI* or other starch biosynthesis or modification genes can be based on the wheat mRNA sequences or derived from homologous DNA or mRNA sequences obtained from other species, for example
25 barley. The antisense sequences may correspond to all or part of the transcripts of any of these genes or for sequences that effect control over their expression, for example their splicing. The antisense sequence may correspond to the targeted coding region of the wheat *SBEIIa* or other gene, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be complementary in part to intron sequences, which may
30 be spliced out during or after transcription, preferably only to exon sequences of the target gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition. In particular embodiments, the length of the antisense sequence is at least 19 contiguous nucleotides, at least 50, at least 100, at least 200, at least 500 or at least 1000 nucleotides corresponding to the
35 complement of the gene RNA sequence. The full-length sequence complementary to the

entire gene transcript may be used. In a particular embodiment, the length of the antisense sequence is 100-2000 nucleotides. In further embodiments, the degree of sequence identity of the antisense sequence to the complement of the targeted transcript is at least 85%, at least 90% or 95-100%. The antisense RNA molecule may of course
5 comprise unrelated sequences which may function to stabilize the molecule.

Cosuppression

Another molecular biological approach that may be used is co-suppression. The mechanism of co-suppression is not well understood but is thought to involve post-
10 transcriptional gene silencing (PTGS) and in that regard may be very similar to many examples of antisense suppression. It involves introducing an extra copy of a gene or a fragment thereof into a plant in the sense orientation with respect to a promoter for its expression. The size of the sense fragment, its correspondence to target gene regions, and its degree of sequence identity to the target gene are as for the antisense sequences
15 described above. In some instances the additional copy of the gene sequence interferes with the expression of the target plant gene. Reference is made to Patent specification WO 97/20936 and European patent specification 0465572 for methods of implementing co-suppression approaches.

20 *Double stranded RNA-mediated gene silencing*

A further method that might be employed to introduce genetic variation into the wheat plant is duplex or double stranded RNA mediated gene silencing. This method also involves PTGS. In this method a DNA is introduced that directs the synthesis of an at least partly double stranded RNA product(s) with homology to the target gene to be
25 inactivated. The DNA therefore comprises both sense and antisense sequences that, when transcribed into RNA, can hybridize to form the double-stranded RNA region. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region that comprises an intron which, when transcribed into RNA, is spliced out. This arrangement has been shown to result in a higher efficiency of gene silencing. The
30 double-stranded region may comprise one or two RNA molecules, transcribed from either one DNA region or two. The presence of the double stranded molecule triggers a response from an endogenous plant system that destroys both the double stranded RNA and also the homologous RNA transcript from the target plant gene, efficiently reducing or eliminating the activity of the target gene. Reference is made to Australian Patent
35 specification 99/29514-A and Patent specification WO 99/53050 for methods of implementing this technique. In particular embodiments, the length of the sense and antisense sequences that hybridise are at least 19 contiguous nucleotides, at least 30, at

least 50, at least 100, at least 200, at least 500 or at least 1000 nucleotides. The full-length sequence corresponding to the entire gene transcript may be used. In a particular embodiment, the lengths are in the range 100-2000 nucleotides. In further embodiments, the degree of sequence identity of the sense and antisense sequences to the targeted transcript is at least 85%, at least 90% or 95-100%. The RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule. The RNA molecule may be expressed under the control of a RNA polymerase II or RNA polymerase III promoter. Examples of the latter include tRNA or snRNA promoters. The double-stranded RNA molecule may also comprise sequences from more than one gene, joined together, and thereby target multiple genes.

Ribozymes

The genetic variation responsible for the desired inactivation of gene expression in wheat may comprise a nucleic acid molecule encoding one or more ribozymes. Ribozymes are RNA molecules with enzymatic or catalytic function that can cleave other RNA molecules at specific sites defined by one or often two hybridizing sequences. The cleavage of the RNA inactivates the expression of the target gene. The ribozymes may also act as an antisense molecule, which may contribute to the gene inactivation. The ribozymes contain one or more catalytic domains, preferably of the hammerhead or hairpin type, between the hybridizing sequences. Other ribozyme motifs may be used including RNaseP, Group I or II introns, and hepatitis delta virus types. Reference is made to European patent specification 0321201 and US Patent No. 6,221,661. The use of ribozymes to inactivate genes in transgenic plants has been demonstrated, for example by Wegener et al (1994).

Genetic constructs/vectors

The invention also provides isolated nucleic acid molecules comprising RNA or DNA, preferably DNA, which encode the gene-inhibiting molecule. In certain embodiments, the nucleic acid molecules encode antisense, sense (co-suppression), double-stranded RNA or ribozyme molecules which target the wheat *SBEIIa* gene sequence and which inactivate its expression in endosperm of wheat grain. The invention also provides genetic constructs comprising or encoding the isolated nucleic acid molecule, comprising one or more regulatory elements such as promoters, enhancers and transcription termination or polyadenylation sequences. Such elements are well known in the art. The genetic constructs may also comprise intron sequences that aid expression of the transgene in plants, particularly in monocotyledonous plants such as wheat. The term "intron" is used in its normal sense as meaning a genetic segment that is transcribed

but does not encode protein and which is spliced out of an RNA before translation. Introns may be incorporated in a 5'-UTR or a coding region if the transgene encodes a translated product, or anywhere in the transcribed region if it does not.

- 5 The invention further provides vectors, for example plasmid vectors, comprising the genetic constructs. The term "vector" includes an expression vector, being capable of *in vitro* or *in vivo* expression, and a transformation vector, capable of being transferred from one cell or organism to another. The vectors comprise sequences that provide for replication in cells, for example in prokaryotic cells such as *E. coli* or *Agrobacterium*. In
10 a particular embodiment, the vector is a binary vector comprising a T-DNA sequence, defined by at least one T-DNA border sequence, that can be introduced into wheat cells. The invention further provides cells comprising the vectors, for example *Agrobacterium* or wheat cells which may be regenerable cells such as the cells of the scutellum of immature embryos. Alternatively, the cells may be transformed wheat cells comprising
15 the transgene.

Promoters/terminators

- In another embodiment, the transgene or other genetic construct of the invention includes a transcriptional initiation region (promoter) that may provide for regulated or
20 constitutive expression in the endosperm of wheat. The promoter may be tissue specific, conferring expression selectively or exclusively in the endosperm. The promoter may be selected from either endosperm-specific (such as High Molecular Weight Glutenin promoter, the wheat SSI promoter, wheat SBEII promoter, wheat GBSS promoter) or promoters not specific for the endosperm (such as ubiquitin promoter or CaMV35S or
25 enhanced 35S promoters). The promoter may be modulated by factors such as temperature, light or stress. Ordinarily, the promoter would be provided 5' of the genetic sequence to be expressed. The construct may also contain other elements that enhance transcription such as the nos 3' or the ocs 3' polyadenylation regions or transcription terminators. The regions of DNA illustrated will be incorporated into
30 vectors containing suitable selectable marker gene sequences and other elements, or into vectors that are co-transformed with vectors containing these sequences.

Transformation methods for wheat

- Methods for transformation of monocotyledonous plants such as wheat, that is for
35 introducing genetic variation into the plant by introduction of an exogenous nucleic acid, and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, Becker et al 1994, Cheng et al 1997, He et al 1994,

Hess et al 1990, Nehra et al 1994, Vasil et al 1992, Vasil et al 1993, Weeks et al 1993, Weir et al 2001, Australian Patent Application No. 75460/94, European Patent Application No. 709462, International Patent Publication Nos. WO93/04178, WO89/12012, WO94/13822 and WO99/14314. Vectors carrying the desired nucleotide
5 sequence or genetic construct and a selectable marker may be introduced into regenerable wheat cells of tissue cultured plants or explants, or suitable plant systems such as protoplasts. The selectable marker gene may provide antibiotic or herbicide resistance to the wheat cells, or allow the utilization of substrates such as mannose. The selectable marker preferably confers asulam, geneticin or hygromycin resistance to the
10 wheat cells. The regenerable wheat cells are preferably from the scutellum of immature embryos, mature embryos, callus derived from these, or the meristematic tissue.

The transformed plant may contain a selectable marker gene, or such gene may be removed during or after regeneration, for example by excision of the selectable marker
15 gene out of the genome or by segregation of the selectable marker gene away from the *SBEIIa*-inhibiting transgene.

Plants where the transgene or mutation has been integrated into a chromosome can be screened for by, for example, using a suitable nucleic acid probe specific for the
20 transgene or phenotypic observation. Any of several methods may be employed to determine the presence of a transformed plant. For example, polymerase chain reaction (PCR) may be used to amplify sequences that are unique to the transformed plant, with detection of the amplified products by gel electrophoresis or other methods. DNA may be extracted from the plants using conventional methods and the PCR reaction carried
25 out using primers that will distinguish the transformed and non-transformed plants. For example, primers may be designed that will amplify a region of DNA from the transformation vector reading into the construct and the reverse primer designed from the gene of interest. These primers will only amplify a fragment if the plant has been successfully transformed. An alternative method to confirm a positive transformant is
30 by Southern blot hybridization, well known in the art. Plants which are transformed or mutant may also be identified i.e. distinguished from non-transformed or wild-type plants by their phenotype, for example conferred by the presence of a selectable marker gene, or the presence of a particular protein by immunological methods, or by the absence of a protein, for example that absence of the *SBEIIa* protein in the endosperm as
35 detected by ELISA assay or Western blot analysis. An indication used in screening such plants might also be by observation of the phenotypic traits of the grain, for

example by visual inspection or measurement of shrunken grain, or testing for elevated amylose content, or checking microscopically for the presence of birefringence.

Mutation

5 Introduction of the genetic variation leading to reduced activity of the *SBEIIa* enzyme or other starch biosynthetic enzyme in the wheat endosperm may also be achieved by the appropriate mutations within the respective gene or regulatory sequences of the gene. In the context of this application, an "induced mutation" is an artificially induced genetic variation which may be the result of chemical, radiation or biologically-based
10 mutagenesis, for example transposon or T-DNA insertion. The extent to which the gene is inhibited will to some degree determine the characteristics of the starch made. The mutations may be truncation or null mutations and these are known to have a significant impact on the nature of the starch, however an altered starch structure will also result from a leaky mutation that sufficiently reduces amylopectin synthesis enzyme activity to
15 provide the characteristic of interest in the starch or grain of wheat. Other chromosomal rearrangements may also be effective and these might include insertions, deletions, inversions, duplication or point mutations. A "null mutation" as used herein refers to a mutation which results in the complete or near complete loss of activity of the gene of interest such as, for example, where the gene activity can no longer be detected.

20 The *SBEIIa* gene is located on the long arm of chromosome 2. It is preferred that mutations to the gene or other genes, particularly deletion mutations, are localised to the gene of interest, for example the *SBEIIa* gene or perhaps extended to the linked *SBEIIb* gene in the case of a double mutant. A gene in this context includes the promoter region and transcription termination/polyadenylation signals as well as the transcribed region.
25 The transcribed region includes the protein coding region(s) and the 5' untranslated and 3' untranslated regions of the mRNA as well as any intron regions that may be present. Mutations to a gene may be in any region of the gene or a combination of regions and might extend from altering only one nucleotide, for example a frameshift mutation in the
30 coding region, to deletion of the entire gene. Plants which are homozygous for the genetic variation are preferred.

Deletions may be restricted in size in the order of one or a few hundred, perhaps 500, kilobases. In certain embodiments, the deletion extends to less than a few thousand
35 kilobases, or less than 5 thousand kilobases. Whilst the invention may encompass larger deletions including much of the long arm of chromosome 2 of the respective

genome these are not preferred because the long arm of chromosome 2 has a number of other genes localised thereon that impact on the vigour of the wheat plant. Accordingly, where large deletions occur, these impact adversely on the vigour of the plant and hence on its commercial viability, and it is desired that at least a majority of the long arm of chromosome 2 is present. In a preferred embodiment, the majority of the long arm of chromosome 2A is present.

- Mutagenesis can be achieved by chemical or radiation means, for example EMS or sodium azide (Zwar and Chandler, 1995) treatment of seed, or gamma irradiation.
- Isolation of mutants may be achieved by screening mutagenised plants or seed. For example, a mutagenized population of wheat may be screened for high amylose content in the grain and/or longer than normal amylopectin chain length distribution, or loss of the SBEIIa protein by ELISA, or for altered grain morphology (Green *et al.*, 1997). Screening is preferably done in a wheat genotype that already lacks one of the SBE activities, for example in a SBEIIb-negative background. Such mutations may then be introduced into desirable genetic backgrounds by crossing the mutant with a plant of the desired genetic background and performing a suitable number of backcrosses to cross out the originally undesired parent background.
- In another embodiment, the mutation affects the expression or activity of both *SBEIIa* and *SBEIIb* genes in wheat. Identifying such a mutation is aided by the unexpected finding that the two genes are closely linked in wheat, in contrast to maize or rice. Deletions in one gene may readily extend to the other gene, providing a null allele (null mutation) for both genes. This knowledge also aids the screening of natural variants that are mutant in both genes on at least one genome of wheat, and more readily allows screening to produce wheat with combined mutations in both genes in two or three genomes. Such wheat provides a high amylose, non-transgenic source of wheat grain and products therefrom.
- Mutations in the genes encoding the SBEIIa or other enzymes involved in amylopectin synthesis will generally cause an increased proportion of amylose content. The amount of amylose per individual grain may be increased as a consequence of diverted carbon flow from amylopectin to amylose, or it may be decreased if there is a significant decrease in starch production per grain. In either case, the relative level of amylose as a percentage of starch increases.

Seed with starch granules having a distorted shape have been reported in high amylose barley (Morell et al, 2003) and in low amylopectin (LAPS) maize having about 90% amylose in starch (Sidebottom et al., 1998).

5 Birefringence is the ability of a substance to refract light in two directions; this produces a dark cross called a "maltese cross" on each starch granule when viewed with a polarizing microscope. Birefringence is an indicator of the degree of ordered structural organization of the polymers within the granules (Thomas and Atwell, 1999). Loss of
10 birefringence in starch granules is generally well correlated with increased amylose content.

Suitable for food production

In another aspect, the invention provides wheat that is useful for food production, the grain having starch comprising a high relative amylose content and a reduced
15 amylopectin content. Preferably the wheat plant from which the grain is obtained has a reduced level of SBEIIa activity in the endosperm during development. The wheat plant of the present invention is useful for food production and in particular for commercial food production. Such food production might include the making of flour, dough or other products that might be an ingredient in commercial food production.

20 The desired genetic background of the wheat will include considerations of agronomic yield and other characteristics. Such characteristics might include whether it is desired to have a winter or spring type of wheat, agronomic performance, disease resistance and abiotic stress resistance. In Australia one might want to cross the altered starch trait into
25 wheat cultivars such as Baxter, Kennedy, Janz, Frame, Rosella, Cadoux, Diamondbird or other commonly grown varieties. The examples provided are specific for an Australian production region, and other varieties will be suited for other growing regions. It is preferred that the wheat variety of the invention provide a yield not less than 80% of the corresponding wild-type variety in at least some growing regions, more preferably not
30 less than 90% and even more preferably not less than 95%. The yield can readily be measured in controlled field trials.

In further embodiments, the starch content of the grain is at least about 25%, 35%, 45%, or 55% to 65% (w/w). Wild-type wheat grown commercially has a starch content
35 usually in the range 55-65%, depending somewhat on the cultivar grown. Alternatively, the grain of the invention has a starch content of at least 90% that of grain from an equivalent, but unaltered, wheat. Lower starch contents than wild-type are likely a

consequence of reduced amylopectin levels. Even with lower starch contents, the grain may still be useful for commercial food production because of the relatively high value of the high amylose products. Other desirable characteristics include the capacity to mill the grain, in particular the grain hardness. Another aspect that might make a wheat plant
5 of higher value is the degree of starch extraction from the grain, the higher extraction rates being more useful. Grain shape is also another feature that can impact on the commercial usefulness of a plant, thus grain shape can have an impact on the ease or otherwise with which the grain can be milled. For example, an elongated grain morphology may make it difficult to mill and process.

10

A fuller grain may be desirable in terms of achieving greater yields and certain benefits of the invention might be achieved, such as the production of starch with high levels of amylose, or in the alternative starch with altered chain length distributions. Thus the grain preferably has a non-shrunken phenotype. Other aspects of the invention may,
15 however, be better achieved by a grain that is less filled. Thus the proportion of aleurone layer or germ or protein to starch may be higher in less filled grain, thereby providing for a wheat flour or other product that is higher in the beneficial constituents of the aleurone layer or protein. The high aleurone layer product might thus be higher in certain vitamins such as folate, or it might be higher in certain minerals such as calcium,
20 and that combined with higher resistant starch levels might provide synergistic effects such as providing for enhanced uptake of minerals in the large bowel.

Starch is readily isolated from wheat grain using standard methods, for example the method of Schulman *et al.* (1991). On an industrial scale, wet or dry milling can be
25 used. Starch granule size is important in the starch processing industry where there is separation of the larger A granules from the smaller B granules. The starch obtained from the grain of wheat plant of the invention has a high relative amylose content.

Physical characteristics of the altered starch

30 Gelatinisation is the heat-driven collapse (disruption) of molecular order within the starch granule in excess water, with concomitant and irreversible changes in properties such as granular swelling, crystallite melting, loss of birefringence, viscosity development and starch solubilisation. High amylose starch from *ae* (amylose extender) mutants of maize showed a higher gelatinisation temperature than normal maize (Fuwa *et al.*, 1999, Krueger *et al.*, 1987). On the other hand, starch from barley *sex6* mutants that
35 lack starch synthase IIa activity had lower gelatinisation temperatures and the enthalpy

for the gelatinisation peak was reduced when compared to that from control plants (Morell et al., 2003).

5 In another aspect of the invention, the starch has an altered gelatinisation temperature as measured by differential scanning calorimetry. This may be either increased or reduced compared to starch from wild-type plants. The altered gelatinisation temperature may be in addition to the relatively high amylose content. The gelatinisation temperature of wild-type wheat starch is typically about 61°C (Rahman et al, 2000) for the temperature of the first peak, defined as the onset temperature, as measured by differential scanning
10 calorimetry.

The starch may also be characterized by its swelling rate in heated excess water compared to wild-type starch. Swelling volume is typically measured by mixing either a starch or flour with excess water and heating to elevated temperatures, typically greater
15 than 90°C. The sample is then collected by centrifugation and the swelling volume is expressed as the mass of the sedimented material divided by the dry weight of the sample. A low swelling characteristic is useful where it is desired to increase the starch content of a food preparation, in particular a hydrated food preparation.

20 The starch structure of the wheat of selected forms of the present invention may also differ in that the degree of crystallinity is reduced compared to normal starch isolated from wheat. The reduced crystallinity of a starch is also thought to be associated with enhance organoleptic properties and contributes to a smoother mouth feel. Thus the starch may additionally exhibit reduced crystallinity resulting from reduced levels of
25 activity of one or more amylopectin synthesis enzymes. Crystallinity is typically investigated by X-ray crystallography.

One measure of an altered amylopectin structure is the distribution of chain lengths, or the degree of polymerization, of the starch. The chain length distribution may be
30 determined by using fluorophore-assisted carbohydrate electrophoresis (FACE) following isoamylase de-branching. The amylopectin of the starch of the invention may have a distribution of chain length in the range from 5 to 60 that is greater than the distribution of starch from wild-type plants upon debranching. Starch with longer chain lengths will also have a commensurate decrease in frequency of branching. Thus the

starch may also have a distribution of longer amylopectin chain lengths in the amylopectin still present.

Food characteristics

5 Starch is the major source of carbohydrate in the human diet, and the grain of the invention and products derived from it can be used to prepare food. The food may be consumed by man or animals, for example in livestock production or in pet-food. The grain derived from the altered wheat plant can readily be used in food processing procedures, and therefore the invention includes milled, ground, kibbled, pearled or
10 rolled grain or products obtained from the processed or whole grain of the wheat plant referred to above, including flour. These products may be then used in various food products, for example farinaceous products such as breads, cakes, biscuits and the like, or food additives such as thickeners or binding agents, or to make drinks, noodles, pasta or quick soups. The grain or products derived from the grain of the invention are
15 particularly desired in breakfast cereals or as extruded products. The high amylose starches of the invention can also be used to form high strength gels that are useful in the confectionery industry, or allow lower molding and curing times. They may also be used as a coating, for example to reduce oil absorption in deep-fried potato or other foods.

20

Dietary fibre

Dietary fibre, in this specification, is the carbohydrate and carbohydrate digestion products that are not absorbed in the small intestine of healthy humans but enter the large bowel. This includes resistant starch, β -glucan and other soluble and insoluble
25 carbohydrate polymers. It is intended to comprise that portion of carbohydrates that are fermentable, at least partially, in the large bowel by the resident microflora.

The starch of the invention preferably contains relatively high levels of dietary fibre, more particularly amylose. The dietary fibre content of the grain of the present invention
30 may or may not result solely from the increased relative endospermal amylose content.

Aspects of this invention might also arise from the combination of aleurone layer and germ in combination with high levels of dietary fibre. Specifically, this may arise where higher relative levels of aleurone or germ are present in the grain. Where the wheat grain
35 is slightly shrunken the endosperm is present in reduced amounts and the aleurone layer and the germ are present in relatively elevated amounts. Thus the wheat has a relatively high level of certain beneficial elements or vitamins in combination with elevated

resistant starch, such elements include divalent cations, bioavailable Ca^{++} and vitamins such as folate or antioxidants such as tocopherols or tocotrienols. One specific form of milled product might be one where the aleurone layer is included in the milled product. Particular milling process might be undertaken to enhance the amount of aleurone layer in the milled product. Thus any product derived from grain milled or otherwise processed to include aleurone layer and germ will have the additional nutritional benefits, without the requirement of adding these elements from separate sources.

Resistant starch

Resistant starch is defined as the sum of starch and products of starch digestion not absorbed in the small intestine of healthy humans but entering into the large bowel. Thus, resistant starch excludes products digested and absorbed in the small intestine. Resistant starches include physically inaccessible starch (RS1 form), resistant granules (RS2), retrograded starches (RS3), and chemically modified starches (RS4). The altered starch structure and in particular the high amylose levels of the starch of the invention give rise to an increase in resistant starch when consumed in food. The starch may be in an RS1 form, being somewhat inaccessible to digestion. Starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant starch

It will be understood that one benefit of the present invention is that it provides for products that are of particular nutritional benefit, and moreover it does so without the need to modify the starch or other constituents of the wheat grain. However it may be desired to make modifications to the starch or other constituent of the grain, and the invention encompasses such a modified constituent. Methods of modification are well known and include the extraction of the starch or other constituent by conventional methods and modification of the starches to increase the resistant form. The starch may be modified by treatment with heat and/or moisture, physically (for example ball milling), enzymatically (using for example α - or β -amylase, pullulanase or the like), chemical hydrolysis (wet or dry using liquid or gaseous reagents), oxidation, cross bonding with difunctional reagents (for example sodium trimetaphosphate, phosphorous oxychloride), or carboxymethylation.

Glycemic index

Glycaemic Index (GI) relates to the rate of digestion of foods comprising the starch, and is a comparison of the effect of a test food with the effect of white bread or glucose on excursions in blood glucose concentration. The Glycaemic Index is a measure of the likely effect of the food concerned on post prandial serum glucose concentration and demand for insulin for blood glucose homeostasis. One important characteristic provided by foods of the invention is a reduced glycaemic index. Furthermore, the foods may have a low level of final digestion and consequently be relatively low-calorie. A low calorific product might be based on inclusion of flour produced from milled wheat grain. Such foods may have the effect of being filling, enhancing bowel health, reducing the post-prandial serum glucose and lipid concentration as well as providing for a low calorific food product.

Non-food applications

The present invention provides modified or improved starches having elevated levels of amylose or reduced levels of amylopectin whose properties satisfy any of various industrial requirements. Starch is widely used in non-food industries, including the film, paper, textile, corrugating and adhesive industries (Young, 1984), for example as a sizing agent. Wheat starch may be used as a substrate for the production of glucose syrups or for ethanol production. The physical properties of unmodified starch limits its usefulness in some applications and often imposes a requirement for chemical modification that can be expensive or have other disadvantages. The invention provides starch for which less post-harvest modification may be required, in particular due to the reduced amylopectin content in combination with other physical properties. For example, the pasting temperature, resistance to shearing stresses, film strength and/or water resistance of starches and product made from the grain of this invention may be altered. The starch may also be used to prepare a biodegradable loose-fill packing material that can be used as a replacement for polystyrene or other packing material.

It will be understood that whilst various indications have been given as to aspects of the present invention, the invention may reside in combinations of two or more aspects of the present invention.

EXAMPLES**EXAMPLE 1. MATERIALS AND METHODS***Carbohydrate determination and analysis*

5 Starch was isolated from wheat grain using the method of Schulman *et al.* (1991).
Starch content was determined using the total starch analysis kit supplied by Megazyme
(Bray, Co Wicklow, Republic of Ireland). The starch content is then compared to
control plants. Subtraction of the starch weight from the total grain weight to give a total
non-starch content of the grain determines whether the reduction in total weight is due to
10 a reduction in starch content.

The amylose content of starch samples was determined by the colorimetric (iodometric)
method of Morrison and Laignelet (1983) with slight modifications as follows.
Approximately 2 mg of starch was weighed accurately (accurate to 0.1 mg) into a 2 ml
15 screw-capped tube fitted with a rubber washer in the lid. To remove lipid, 1 ml of
85% (v/v) methanol was mixed with the starch and the tube heated in a 65°C water
bath for 1 hour with occasional vortexing. After centrifugation at 13,000g for 5 min,
the supernatant was carefully removed and the extraction steps repeated. The starch
was then dried at 65°C for 1 hour and dissolved in urea-dimethyl sulphoxide solution
20 (UDMSO; 9 volumes of dimethyl sulphoxide to 1 volume of 6 M urea), using 1 ml of
UDMSO per 2 mg of starch (weighed as above). The mixture was immediately
vortexed vigorously and incubated in a 95°C water bath for 1 hour with intermittent
vortexing for complete dissolution of the starch. An aliquot of the starch-UDMSO
solution (50 µl) was treated with 20 µl of I₂-KI reagent that contained 2 mg iodine and
25 20 mg potassium iodide per ml of water. The mixture was made up to 1 ml with water.
The absorbance of the mixture at 650 nm was measured by transferring 200 µl to
microplate and reading the absorbance using an Emax Precision Microplate Reader
(Molecular Devices, USA). Standard samples containing from 0 to 100% amylose and
100% to 0% amylopectin were made from potato amylose and corn (or potato)
30 amylopectin (Sigma) and treated as for the test samples. The amylose content
(percentage amylose) was determined from the absorbance values using a regression
equation derived from the absorbances for the standard samples. Analysis of the
amylose/amylopectin ratio of non-debranched starches may also be carried out
according to Case *et al.*, (1998) or by an HPLC method for separating debranched
35 starches as described by Batey and Curtin (1996).

The distribution of chain lengths in the starch may be analysed by fluorophore assisted carbohydrate electrophoresis (FACE) using a capillary electrophoresis unit according to Morell *et al* (1998), after debranching of the starch samples. The gelatinisation temperature profiles of starch samples may be measured in a Pyris 1 differential scanning calorimeter (Perkin Elmer, Norwalk CT, USA). The viscosity of starch solutions may be measured on a Rapid-Visco-Analyser (RVA, Newport Scientific Pty Ltd, Warriewood, Sydney), for example using conditions as reported by Batey *et al*, 1997. The parameters that may be measured include peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature. The swelling volume of flour or starch may be determined according to the method of Konik-Rose *et al* (2001). The uptake of water is measured by weighing the sample prior to and after mixing the flour or starch sample in water at defined temperatures and following collection of the gelatinized material.

15 β -Glucan levels may be determined using the kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland).

Analysis of protein expression in endosperm.

Specific protein expression in endosperm was analyzed by Western blot procedures.

20 Endosperm was dissected away from all maternal tissues and samples of approximately 0.2 mg were homogenized in 600 μ l of 50 mM KPi buffer (42 mM K_2HPO_4 and 8 mM KH_2PO_4), pH 7.5, containing 5 mM EDTA, 20% glycerol, 5 mM DTT and 1 mM Pefabloc. The ground samples were centrifuged for 10 min at 13,000g and the supernatant aliquoted and frozen at $-80^{\circ}C$ until use. For total protein estimation, a BSA standard curve was set up using 0, 20, 40, 60, 80 and 100 μ l aliquots of 0.25 mg/ml BSA standard. The samples (3 μ l) were made up to 100 μ l with distilled water and 1 ml of Coomassie Plus Protein reagent was added to each. The absorbance was read after 5 min at 595nm, using the zero BSA sample from the standard curve as the blank, and the protein levels in the samples determined. Samples containing 20 μ g total protein from

25 each endosperm were run on an 8% non denaturing polyacrylamide gel containing 0.34 M Tris-HCl (pH 8.8), acrylamide (8.0%), ammonium persulphate (0.06%) and TEMED (0.1%). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane according to Morell *et al*, (1997) and immunoreacted with SBEIIa or SBEIIb specific antibodies.

30

EXAMPLE 2. GENETIC CONSTRUCTS FOR THE ALTERATION OF WHEAT *SBEIIa* AND *SBEIIb* EXPRESSION.

5 Duplex-RNA (dsRNA) constructs were made to reduce the expression of either the *SBEIIa* or *SBEIIb* genes of wheat. In such constructs, the desired nucleic acid sequence corresponding to part of the *SBEIIa* or *SBEIIb* genes occurred in both the sense and antisense orientations relative to the promoter so that the expressed RNA comprised complementary regions that were able to basepair and form a duplex or double-stranded
10 RNA. A spacer region between the sense and antisense sequences comprised an intron sequence which, when transcribed as part of the RNA in the transformed plant, would be spliced out to form a tight "hairpin" duplex structure. The inclusion of an intron has been found to increase the efficiency of gene silencing conferred by duplex-RNA constructs (Smith et al, 2000). The desired nucleic acid was linked to a high molecular
15 weight glutenin (HMWG) promoter sequence (promoter of the Dx5 subunit gene, Accession No. X12928, Anderson et al., 1989) and terminator sequence from the nopaline synthase gene from *Agrobacterium* (nos3'). This provided endosperm specific expression of the dsRNA sequences.

20 The *SBEIIa* duplex-RNA construct contained 1536bp of nucleotide sequence amplified by PCR from the wheat *SBEIIa* gene (GenBank Accession number AF338431, see Figure 1). This included; a 468bp sequence that comprises the whole of exons 1 and 2 and part of exon 3 (nucleotide positions 1058 to 1336, 1664 to 1761 and 2038 to 2219 in Figure 1), with *EcoRI* and *KpnI* restriction sites on either side (fragment 1), a 512bp
25 sequence consisting of part of exons 3 and 4 and the whole of intron 3 of *SBEIIa* (nucleotide positions 2220 to 2731 in Figure 1) with *KpnI* and *SacI* sites on either side (fragment 2) and a 528bp fragment consisting of the complete exons 1, 2 and 3 of *SBEIIa* (nucleotide positions 1058 to 1336, 1664 to 1761 and 2038 to 2279 in Figure 1) with *BamHI* and *SacI* sites on either side (fragment 3). Fragments 1, 2 and 3 were then
30 ligated so that the sequence of fragment 3 was ligated to fragment 2 in the antisense orientation relative to fragment 1. The duplex-RNA constructs were initially generated in the vector pDVO3000 which contains the HMWG promoter sequence and nos3' terminator. The gene construct in the vector pDVO3000 was designated pDVO3-IIa and the duplex-RNA gene designated ds-*SBEIIa*.

35

The strategy for the *SBEIIb* duplex-RNA construct was similar. The *SBEIIb* construct contained a fragment of 1607bp amplified by PCR from the wheat *SBEIIb* gene

(sequence is outlined in Figure 2). This included a 471bp sequence that comprised the whole of exons 1 and 2 and part of exon 3 (nucleotide positions 489 to 640, 789 to 934 and 1598 to 1769 in Figure 2), with *EcoRI* and *KpnI* restriction sites on either side (fragment 1), a 589bp sequence consisting of part of exons 3 and 4 and the whole of intron 3 of *SBEIIb* (nucleotide positions 1770 to 2364 in Figure 2) with *KpnI* and *SacI* sites on either side (fragment 2) and a 528bp fragment consisting of the complete exons 1, 2 and 3 of *SBEIIb* (nucleotide positions 489 to 640, 789 to 934 and 1598 to 1827 in Figure 2) with *BamHI* and *SacI* sites on either side (fragment 3). Fragments 1, 2 and 3 were then ligated so that the sequence of fragment 3 was ligated to fragment 2 in the antisense orientation relative to fragment 1. The *SBEIIb* duplex-RNA gene construct in the vector pDVO3000 was designated pDVO3-IIb and the duplex-RNA gene designated ds-*SBEIIb*. The constructs are shown schematically in Figure 3.

Each of the ds-RNA expression cassettes was then cut out with the restriction enzyme *XhoI* and inserted into the binary transformation vectors pGB53 and pBIOS340. pGB53 was created from pSB11 (Komari et al., 1996) by the introduction of the gene encoding asulam resistance (*sul*) driven by the rice actin promoter, leaving a unique *XhoI* site adjacent to the right T-DNA border for the introduction of a gene of interest. Similarly, pBIOS340 was created from pSB1 (Komari et al., 1996) by the introduction of an *nptII* gene encoding kanamycin and geneticin resistance, driven by the rice actin promoter, again leaving a unique *XhoI* site adjacent to the right border. The *SBEIIa* constructs in pGB53 and pBIOS340 were designated pCL51 and pCL59, respectively, and the *SBEIIb* constructs in pGB53 and pBIOS340 were designated pCL54 and pCL60, respectively.

EXAMPLE 3: TRANSFORMATION OF WHEAT.

Genetic constructs for transformation of wheat were introduced by electroporation into the disarmed *Agrobacterium tumefaciens* strain LBA4404 carrying the *vir* plasmid pAL4404 and pSB1, with subsequent selection on media with spectinomycin. Transformed *Agrobacterium* strains were incubated on solidified YEP media at 27°C for 2 days. Bacteria were then collected and re-suspended in TSIM1 (MS media with 100 mg/l myo-inositol, 10 g/l glucose, 50 mg/l MES buffer pH5.5) containing 400 mM acetosyringone to an optical density of 2.4 at 650 nm for wheat inoculation.

Wheat plants (variety NB1, a Spring wheat variety obtained from Nickerson Seeds Ltd, Rothwell, Lincs.) were grown in a glasshouse at 22/15°C day/night temperature with

- supplemented light to give a 16 hour day. Tillers were harvested approximately 14 days post-anthesis (embryos approximately 1 mm in length) to include 50 cm tiller stem. All leaves were then removed from the tillers except the flag leaf, which was cleaned to remove contaminating fungal spores. The glumes of each spikelet and the lemma from the first two florets were then carefully removed to expose the immature seed. Generally, only these two seed in each spikelet were uncovered. This procedure was carried out along the entire length of the inflorescence. The ears were then sprayed with 70% IMS as a brief surface sterilization.
- 10 *Agrobacterium* suspensions (1 μ l) were inoculated using a 10 μ l Hamilton syringe into the immature seed approximately at the position of the scutellum: endosperm interface so that all exposed seed were inoculated. The tillers were then placed in water, covered with a translucent plastic bag to prevent seed dehydration, and placed in a lit incubator for 3 days at 23°C, 16 hr day, 45 μ mol m⁻² s⁻¹ PAR. After 3 days of co-cultivation, the inoculated
- 15 immature seed were removed and surface sterilized with 70% ethanol (30 sec), then 20% bleach (Domestos, 20 min), followed by thorough washing in sterile distilled water. Immature embryos were aseptically isolated and placed on W3 media (MS supplemented with 20 g/l sucrose and 2 mg/l 2,4-D and solidified with 6 g/l Type I agarose, Sigma) with the addition of 150mg/l Timentin (W3T) and with the scutellum
- 20 uppermost (20 embryos per plate). Cultures were placed at 25°C in the light (16 hour day, 80 μ mol m⁻² s⁻¹ PAR). The development of the embryonic axis on the embryos was assessed about 5 days after isolation and the axis was removed where necessary to improve callus production. The embryos were maintained on W3T for 4 weeks, with a transfer to fresh media at 2 weeks post-isolation and assessed for embryogenic capacity.
- 25 After 4 weeks growth, callus derived from the inoculated embryos was very similar to control callus obtained from uninoculated embryos plated on W3T medium. Presence of the bacteria did not appear to have substantially reduced the embryogenic capacity of the callus derived from the inoculated embryos. Embryogenic calli were transferred to
- 30 W3 media with 2 mg/l Asulam (where pGB53 derivatives were used) or geneticin at 25 mg/l (pBIOS340 derivatives) and 150mg/l Timentin (W32AT). Calli were maintained on this media for a further 2 weeks and then each callus was divided into 2 mm-sized pieces and re-plated onto W32AT. Control embryos derived from inoculations with the LBA4404 without binary vector constructs did not produce transformed callus on
- 35 selection media.

After a further 2 weeks culture, all tissue was assessed for development of embryogenic callus: any callus showing signs of continued development after 4 weeks on selection was transferred to regeneration media (RMT – MS with 40 g/l maltose and 150 mg/l Timentin, pH 5.8, solidified with 6 g/l agarose, Sigma type 1). Shoots were regenerated within 4 weeks on this media and then transferred to MS30 with 150 mg/l Timentin for shoot elongation and rooting. Juvenile plants were then transferred to soil mixture and kept on a misting bench for two weeks and finally transferred to a glasshouse.

A total of 3217 embryos using pCL54 or pCL60 (ds-SBEIIb) and 2010 embryos using pCL51 or pCL59 (ds-SBEIIa) were treated by this method and 61 plants were regenerated from calli for the IIb transformation and 31 plants regenerated from calli for the IIa transformation. Survival on selection medium suggested that they were successfully transformed with the gene construct. A large majority, but not all, of the plants that were transformed with the selectable marker gene would be expected to integrate the SBEIIa or SBEIIb inhibitory gene; these could readily be distinguished as described in the following examples.

The recovery of multiple, stable integration events with good regeneration potential from the experiments indicated that the seed inoculation transformation method used here was as efficient as other reported methods for wheat. Alternative *Agrobacterium* strains such as strain AGL1 or selectable markers such as genes encoding hygromycin resistance can also be used in the method.

EXAMPLE 4. ANALYSIS OF WHEAT TRANSFORMANTS.

Transformation was determined by one or more of the following methods:
 PCR analysis for one or more of the transgenes. PCR analysis was performed on genomic DNA extracted from 1-2 cm² of fresh leaf material using the mini-prep method described by Stacey and Isaac (1994). PCR reactions were performed, for example, using the primers SBEIIa-For: 5'- CCGCTGCTTTCGCTCATTTTG-3' [SEQ ID No. 9] and SBEIIa-Rev: 5'-GACTACCGGAGCTCCACCTTC-3' [SEQ ID No. 10] designed to amplify a fragment (462bp) from the *SBEIIa* gene, or SBEIIb-DupFor 5'-AGATGTGAATGGCTGCTTGCTG-3' [SEQ ID No. 11] and SBEIIb-DupRev 5'-CAGGTCGACCATATGGGAGAGC-3' [SEQ ID No. 12] for *SBEIIb* (505bp).
 Reaction conditions were as follows: "hot start" (94°C, 3 min) followed by 30 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), extension (73°C, 2 min) followed by 1 cycle at 73°C (5 min).

Southern blot hybridization analysis is performed on DNA from a larger scale (9 ml) extraction from lyophilized ground tissue (Stacey and Isaac, 1994). DNA samples are adjusted to 0.2 mg/ml and digested with restriction enzymes such as *HindIII*, *EcoRI* and
5 *KpnI*. Restriction enzyme digestion, gel electrophoresis and vacuum blotting are carried out as described by Stacey and Isaac (1994). Digoxigenin-labelled probes including the intron 3 region of the ds-SBEII constructs are produced by PCR according to the method of McCreery and Helentjaris (1994). Hybridization of the probes to the Southern blot and detection by chemiluminescence are performed according to the
10 method of McCreery and Helentjaris (1994).

The results of the PCR analyses are summarized in Table 2. Plants that were positive for the transgenes as demonstrated by PCR included 27 independent transformation events for ds-SBEIIa and 61 independent events for ds-SBEIIb.

Table 2. Transformation of wheat with SBEIIa and SBEIIb RNA duplex constructs.

Experiment No.	No. of embryos inoculated	No. of lines regenerated	PCR positive lines
ds-SBEIIa construct			
44	242	1	1
50	169	3	3
52	158	3	3
58	163	2	2
61	195	1	1
72	185	1	0
83	241	1	1
84	242	1	1
85	153	5	5
109	262	13	10
Total	2010	31	27
ds-SBEIIb construct			
48	291	1	1
51	166	1	0
53	194	1	0
55	261	1	1
59	253	1	0
60	175	4	2
62	199	1	0
70	152	1	0
73	238	2	2
75	151	2	2
76	150	1	0
77	150	2	2
81	134	1	1
87	230	5	3
92	233	8	5
110	240	29	16
Total	3217	61	35

EXAMPLE 5. ANALYSIS OF GRAIN FROM PLANTS TRANSFORMED WITH DUPLEX-RNA CONSTRUCTS.

5 Starch granule morphology.

The morphology of starch granules from mature T1 seed obtained from the T0 transformed wheat plants was observed by light microscopy. Ten individual grains from each of 25 T0 plants independently transformed with ds-SBEIIa and 12 plants independently transformed with ds-SBEIIb were analysed. Each endosperm was gently crushed to release the starch granules, which were dispersed in water and visualized under a light microscope. Of the 25 ds-SBEIIa lines analysed, 12 had grains with distorted granules (for example, see Figure 4) although the visual observation revealed varying levels of distortion in different seeds. In contrast, none of the 12 ds-SBEIIb lines showed significant starch granule distortion in the endosperm when observed under light microscopy. The results are summarized in Tables 3 and 4.

Table 3. Starch granule morphology of T1 seeds of ds-SBEIIa transgenic wheat lines.

Slide No.	Line No	Granule Morphology*
1	44.1a	+
2	50.1b	-
3	50.2b	+
4	50.3x	-
5	52.1a	+
6	52.2a	+
7	52.3a	+/-
8	58.1a	-
9	58.2a	-
10	61.2a	-
11	83.1b	+
12	84.1a	+/-
13	85.1a	+/-
14	85.2c	-
15	85.3a	-
16	85.4b	+
17	85.5a	-
18	109.1a	-
19	109.2c	+
20	109.3b	+
21	109.4e	-

54

22	109.7b	-
23	109.8c	-
24	109.10a	+
25	109.11x	+

5 * Starch granule morphology of 10 seeds from each line was observed.
Morphology is indicated as + if all the ten seeds had normal granule morphology, -
if there were seeds which were severely distorted, and +/- for some abnormality, i.e.
at least some seeds with some distortion but none with severe distortion.

Table 4. Starch granule morphology of T1 seeds of ds-SBEIIb transgenic wheat lines.

10

Slide No	Lines	Granule Morphology
1	48.1a	+
2	55.1a	+
3	60.1a	+
4	60.4a	+
5	73.1f	+
6	75.1c	+
7	75.3x	+
8	77.1c	+
9	77.2c	+
26	110.16b	+
27	110.17b	+
28	110.18a	+

+ indicates that all the ten seeds from each line had normal starch granule morphology

15 Observing the starch granules under polarized light revealed that there was a significant
reduction in birefringence for distorted granules (Figure 5) for the ds-SBEIIa grain.
Loss of birefringence was observed for 94% of the granules in seeds from the line
50.1b, correlating with their distorted phenotype, while normal granules from another
seed of the same line showed full birefringence (Table 5). The seed with normal
granules is presumed to be a segregant lacking the transgene and therefore
20 phenotypically normal.

Table 5. Birefringence of starch granules from T1 seeds of the ds-SBEIIa transgenic wheat line 50.1b

5

Seed from the line 50.1b	Microscopic field	No. of granules showing no BF	No. of granules showing partial BF	No. of granules showing full BF
Seed with distorted granules	1	55	2	1
	2	73	1	0
	3	44	1	0
	4	92	3	2
	5	46	2	7
	6	46	2	3
Total		356 (93.7%)	11 (2.9%)	13 (3.4%)
Seed with normal granules	1	1	3	110
	2	1	1	38
	3	3	4	90
	4	0	3	61
	5	3	3	59
	6	1	0	30
Total		9 (2.2%)	14 (3.4%)	388 (94.4%)

10 Light microscopy results are confirmed by scanning electron microscopy (SEM) of the starch granules. To do this, purified starch is sputtered with gold and scanned at 15 kV at room temperature.

Grain weight.

15 Individual grains from ds-SBEIIa transformed plants, grown under equivalent conditions in the greenhouse, were weighed (Table 6). Grains having severely distorted granules from plants 50.1b, 58.2a, 61.2a and 109 were not significantly reduced in average weight compared to grains of wild-type plants grown under the same conditions. Therefore, starch production did not appear to be substantially reduced even in the seeds with highly distorted starch granules. This data also suggests that the yield of field-grown
20 wheat with reduced SBEIIa activity in the endosperm is about normal.

Table 6. Grain weight of T1seeds from the ds-SBEIIa transgenic wheat lines

Transgenic Line	Seed No	Seed weight (mg)	Starch granule morphology*	Transgenic Line	Seed No	Seed weight (mg)	Starch granule morphology*
50.1b	1	16.9	+	61.2a	1	50.7	+

	2	49.8	+		2	49.0	+/-
	3	46.9	-		3	49.8	-
	4	50.0	-		4	47.0	-
	5	45.4	-		5	48.6	-
	6	42.6	-		6	46.2	-
	7	39.9	+/-		7	42.2	+
	8	41.0	+		8	50.4	-
	9	39.5	-		9	39.7	-
	10	37.0	+/-		10	46.3	-
58.2a	1	44.0	-	109.7b	1	40.1	-
	2	37.4	+		2	34.6	-
	3	48.8	-		3	43.7	-
	4	43.2	+		4	38.8	-
	5	46.2	-		5	33.8	+/-
	6	42.1	+		6	31.1	+/-
	7	43.5	+/-		7	35.9	+
	8	45.7	-		8	44.3	+/-
	9	38.8	-		9	37.7	-
	10	38.1	+/-		10	41.4	-

+ normal starch granules, - severely distorted granules, +/- mild distortion of granules

Analysis of SBEIIa and SBEIIb proteins in T2 transgenic wheat endosperm.

- 5 Seed (T2) from 13 ds-SBEIIa transformed T1 plants, representing 5 independently transformed lines, and from 9 ds-SBEIIa transformed plants, representing 3 independently transformed lines, were analysed for SBEIIa and SBEIIb protein expression in endosperm by non denaturing PAGE and Western blotting. The ds-SBEIIa plants were all from lines having abnormal starch granule morphology, while the
- 10 ds-SBEIIb lines all had normal granule morphology, as described above. The antibody used for detection of SBEIIa was 3KLH, from rabbits, which had been raised against the synthetic peptide having the amino acid sequence AASPGKVLVPDESDDLGC [SEQ ID No. 13], corresponding to the sequence from the N-terminus of mature SBEIIa, and was diluted 1:5000 for use. The antibody used for detection of SBEIIb was R6, raised
- 15 against the synthetic peptide having the amino acid sequence AGGPSGEVMIGC [SEQ ID No. 14], corresponding to the deduced sequence from the N-terminus of mature SBEIIb and diluted 1:6000 before use. The secondary antibody used was GAR-

Horseradish Peroxidase conjugate (1:3000 dilution). Immunoreactive bands were revealed using an Amersham ECL-detection system.

Endosperms from each of seven developing grains (15 days post anthesis) from each of the 22 T1 plants were analysed as it was expected that some of the plants would be heterozygous for the transgene. Twelve of the 13 ds-SBEIIa plants produced T2 progeny showing reduced levels of SBEIIa protein in the endosperm. All seven seeds from one line (50.3x.9) appeared to lack SBEIIa entirely, while all seven seeds from four other plants showed obviously reduced expression of SBEIIa (Table 7). These could represent lines that are homozygous for the transgene. Seven lines were segregating for the absence of SBEIIa or reduced levels of SBEIIa, or in some cases no apparent reduction of the protein, and these lines probably represent heterozygotes for the transgene. The thirteenth line (50.3x.6) was homozygous for wild type expression (Table 7).

Table 7. Western blot analysis of endosperm proteins from T2 ds-SBEIIa transgenic wheat lines

Transgenic line	Gene targeted	T1 granule morphology	Segregation of SBEII protein band in T2 grains
50.3x.6	SBEIIa	+	Uniform for wild type expression (+)
58.1a.3	"	-	Segregating for +/- and -
58.1a.7	"	-	Segregating for +, +/- and -
58.1a.9	"	-	Segregating for +, +/- and -
50.1b.3	"	-	Uniform for +/-
50.1b.4	"	-	Segregating for +/- and -
50.1b.5	"	-	Uniform for +/-
50.1b.9	"	-	Segregating for + and +/-
50.3x.9	"	-	Uniform for -
61.2a.8	"	-	Uniform for +/-
61.2a.9	"	-	Segregating for +/- and -
61.2a.10	"	-	Uniform for +/-
85.2c.2	"	-	Segregating for +/- and -
110.16b.14	SBEIIb	+	Uniform for wild type expression (+)
110.16b.2	"	+	Uniform for -
110.16b.17	"	+	Uniform for +
110.16b.5	"	+	Uniform for -
110.16b.19	"	+	Uniform for -
110.17b.3	"	+	Segregating for +/- and -
110.17b.6	"	+	Segregating for + and +/-

110.18a.9	“	+	Segregating for +/- and -
110.18a.17	“	+	Segregating for +, +/- and -

Of the nine ds-SBEIIb transgenic lines tested, three (110.16b.2, 110.16b.5 and 110.16b.19) uniformly showed no SBEIIb expression in each of seven progeny seeds, while two were uniform for wild type expression and the remaining four were

5 segregating for no expression, reduced expression or wild-type (Table 7). Embryos from the seeds may be grown (embryo rescue) to produce T2 plants and T3 seed which are screened by PCR and protein expression analysis to confirm the genetic status of the T2 seed with respect to the transgene.

10 These data indicate that the duplex-RNA constructs are effective in reducing the expression of the *SBEIIa* and *SBEIIb* genes in endosperm of wheat. The data also indicate that reduction of *SBEIIb* expression alone did not substantially alter starch granule morphology.

15 The expression of the *SBEIIb* gene in transgenic seeds containing the ds-SBEIIa transgene and lacking SBEIIa protein, and the expression of the *SBEIIa* gene in seeds containing the ds-SBEIIb were also analyzed by the Western blot method. Unexpectedly, transgenic seeds comprising ds-SBEIIa were much reduced for SBEIIb. However, the converse effect was not observed in seeds transgenic for ds-SBEIIb. The

20 SBEIIa expression was unaltered in the seeds in which SBEIIb was completely silenced by ds-SBEIIb. It is possible that expression of *SBEIIb* was suppressed by the ds-SBEIIa construct due to sequence homology between the genes in the region used for the duplex construct, it is also possible that the activity of SBEIIb was reduced by the ds-SBEIIa transgene by some other mechanism.

25

The expression levels of the *SBEIIa* and *SBEIIb* genes can also be specifically determined at the mRNA levels through standard techniques such as Northern hybridisation or RT-PCR methods, for example by using probes from non conserved regions or primer pairs which hybridize to unique sites in one of the genes but not the

30 other, for example in the 3' untranslated regions. Such regions or sites can readily be identified by comparison of the two gene sequences.

EXAMPLE 6. STARCH ANALYSIS OF TRANSFORMED WHEAT.

35 *Amylose and amylopectin levels in transgenic wheat grain.*

The amylose content of starches from six pooled T1 seed samples was determined as described in Example 1. The pooled seed samples were obtained from the transgenic wheat lines as follows:

- Pool 1- seed that had distorted starch granules from the ds-SBEIIa transgenic line
 5 85.2c
 Pool 2- seed that had normal granules from the ds-SBEIIa transgenic line 85.1a
 Pool 3- seed that had normal granules from the ds-SBEIIb transgenic line 110.18a
 Pool 4- seed that had distorted granules from the ds-SBEIIa transgenic lines 58.1a,
 58.2a and 61.2a, pooled together
 10 Pool 5- seed that had normal granules from the ds-SBEIIa transgenic line 83.1b
 Pool 6- seed that had normal granules from the ds-SBEIIb transgenic line 75.3x.

Each analysis was done using four replicates of the starch samples. The regression equation used to convert the absorbance to amylose content for these analyses was
 15 $Y=57.548x-8.793$, where Y was the amylose content (%) and x was the absorbance.

The results are given in Table 8. The presence of distorted starch granules was clearly associated with increased amylose contents. Starch from grains with distorted granules from the ds-SBEIIa transgenic lines (pools 1 and 4) had an amylose content of greater
 20 than 50% while the other starch pools, derived from grain with normal starch granules, had amylose contents in the range 21-26%. This included starch from line IIb 110.18a which had reduced expression of SBEIIb (Table 8), which suggests that inactivation of *SBEIIb* alone in wheat does not substantially increase amylose levels in grain starch.

25

Table 8. Amylose content estimated by iodometric method of the transgenic wheat lines

Starch sample	Transgenic line	Amylose content (%)			
		Replication 1	Replication 2	Replication 3	Mean
Pool 1	85.2c	65.7	54.2	53.2	57.7
Pool 2	85.1a	23.7	22.5	26.7	24.3
Pool 3	110.18a	22.3	21.0	21.5	21.6
Pool 4	58.1, 58.2a, 61.2a	53.9	52.8	58.5	55.1

60

Pool 5	83.1b	26.5	25.3	24.8	25.6
Pool 6	75.3x	24.3	20.6	19.5	21.5

A second set of analyses was done by the iodometric method using a sample from Pool 4 and starch from wheat that is defective in *SSII* (Yamamori et al. 2000) and from barley line M292 which is mutant in *SSIIa*. The amylose content determined for starch from Pool 4 wheat seeds (ds-SBEIIa transgenic lines) was considerably higher than that of starch from the *SSII* mutants of wheat and barley.

This implies that the amylopectin content in the starch of these grains is considerably reduced, from about 75% in wild-type to less than 50% or even less than 20%.

Lines containing both ds-SBEIIa and ds-SBEIIb transgenes are generated by crossing the transgenic plants described above. Amylose contents in the grain starch of such progeny are elevated above that for starch from plants containing only ds-SBEIIa, for example to 75 or 80%, showing that inhibition of SBEIIb in addition to SBEIIa further elevates amylose levels.

EXAMPLE 7. COMPARISON OF SBEIIa FROM A, B AND D GENOMES.

Construction of wheat cDNA and genomic libraries.

Wheat endosperm cDNA and genomic libraries were made by standard methods in phage vectors (Sambrook et al, 1989). Two cDNA libraries were made, one from RNA from the cultivar Rosella (Rahman et al., 1999) and the other from cultivar Wyuna (Rahman et al. 2001). The Rosella library was in the vector ZAPII and used *EcoRI* and *NotI* primers while the Wyuna library was in the ZipLox vector (Life Technology) according to the protocols supplied with the reagents. The titres of the libraries were 2×10^6 pfu tested with Y1090(ZL) strain of *E. coli*. A genomic library was made from DNA from *A. tauschii* variety 10097. The DNA was digested with *Sau3A* and ligated to partially filled lambdaGEM12 vector (Promega). Cloned fragments could be released with *SacI* or *XhoI* digestion. Genomic libraries of *T. aestivum* DNA were as described by Turner et al. (1999).

Isolation of SBEIIa cDNA sequences.

Using a wheat SBEI gene sequence probe at low stringency (Rahman et al. 2001), cDNAs were isolated from the library prepared from the cultivar Rosella. The longest clone obtained, designated sbe9 was sequenced and seen to encode a SBEIIa type of

sequence (Genbank AF338432.1). Subsequently, three clones were isolated from the endosperm cDNA library prepared from the cultivar Wyuna (Rahman et al. 2001) using a probe corresponding to positions 536 to 890 of *sbe9*. Conditions for library screening were hybridisation at 25% formamide, 5 x SSC, 0.1% SDS, 10x Denhardt's solution,
 5 100 µg/ml salmon sperm DNA at 42°C for 16hr, followed by washing with 2 x SSC, 0.1% SDS at 65°C for 3 x 1hr (medium stringency). Three different sequences were obtained by sequencing the clones and these are represented below by sr995 and sr997 (Figure 6).

10 Investigation of these cDNA sequences indicated that different sequences were expressed in the wheat endosperm and these are likely to correspond to *SBEIIa* transcripts from the different wheat genomes. A PILEUP comparison of the sequences with other known wheat *SBEIIa* cDNA sequences showed that sr995 and sr996 sequences clustered with the mRNA sequence derived from the D-genome sequence
 15 wSBE-D1 (sr854) (Figure 7), suggesting that sr995 and sr996 represent transcripts from the D genome *SBEIIa*. Sr997 clustered with the Y11282 sequence (Nair et al., 1997) indicating that they are probably from the same genome, either the A or B genome. The previously described *sbe9* (AF338432.1) is probably from the same genome as Y11282 but represents an alternative splicing event, consistent with one exon
 20 near the 5' end being spliced out.

Distinguishing SBEIIa genes from the A, B and D genomes of wheat T. aestivum.

Differences in the gene or RNA transcript sequences may be used as a basis of designing A, B and D genome specific primers for mutational screening either at the gene level or
 25 at the RNA level. For example, Figure 6 compares *SBEIIa* nucleotide sequences from cDNAs including Genbank accession Y11282, and partial sequences of cDNAs *sbe9* (AF338432.1), sr997 and sr995. Genomic sequences are available for *SBEIIa* genes from *T. aestivum*, for example see Table 1. Genomic sequences have been ascribed to the A, B and D genomes. Comparison shows polymorphisms, any of which can be used
 30 to distinguish the sequences by molecular means.

A forward primer based on a region of exon 5 (5'-ATCACTTACCGAGAATGGG-3') [SEQ ID No. 15] and a reverse primer based on a sequence in exon 6 (5'-CTGCATTTGGATTCCAATTG-3') [SEQ ID No. 16] have been used to distinguish
 35 between products from the A, B and D genomes. Such primers may be used in PCR

reactions to screen for what varieties or accessions that are mutant in one or more of the *SBEIIa* genes from the A, B or D genomes (see below).

5 PCR based markers have also been developed to distinguish the *SBEIIb* genes from the A, B and D genomes of wheat. For example, PCR reactions with the primer pair ARA19F (5'-CACCCATTGTAATTGGGTACACTG-3') [SEQ ID No. 17] and ARA15R (5'-TCCATGCCTCCTTCGTGTTTCATCA-3') [SEQ ID No. 18] followed by digestion of the amplification products with the restriction enzyme *RsaI* could distinguish the *SBEIIb* genes from the three genomes.

10

The differences in cDNA sequence are reflected in the deduced protein sequences. For example, the deduced full-length amino acid sequences for the D genome (sr854) and A or B genome (Y11282) polypeptides are compared in Figure 8. Significant differences are evident at regions 688-698 and 735-6 which could be used for producing genome-specific antibodies to the *SBEIIa* proteins, in order to screen for wheat varieties lacking one or more genome-specific activities. Other differences occur in the transit peptide sequences which correspond to amino acid positions 1-54 of Figure 8.

15

20 **EXAMPLE 8. IDENTIFICATION OF WHEAT VARIETIES MUTANT IN ONE OR MORE *SBEII* GENES.**

*Identification of *SBEIIb* null mutations in B and D genomes*

A total of 1500 wheat accessions including 300 Australian wheat varieties, 900 wheat accessions from the Australian Winter Cereal Collection (AWCC, Tamworth, NSW Australia) and 300 wheat land races were screened by PCR amplification of an *SBEIIb* marker, corresponding to a polymorphic intron 3 region, using the primers ARA19F (see above) and ARA23R (5'-CTGCGCATAAATCCAAACTTCTCG-3') [SEQ ID No. 19]. PCR amplification used conditions as described above. Amplification products were digested with the restriction enzyme *RsaI* and electrophoresed on polyacrylamide gels. Three lines (Aus12745, Aus17340 and Aus10103) lacked the D genome marker and two lines (Aus12509 and Aus12565) lacked the B genome marker (Figure 9). These lines represent presumed null mutants in the *SBEIIb* genes for the B or the D genomes.

25

30

35 Southern blot hybridization analysis was carried out on DNA from the null mutant lines to confirm the PCR results. *HindIII* digested DNA, prepared from the plants by standard methods, was electrophoresed on 1% agarose gels and blotted on to Hybond

N+ nylon membrane (Amersham). Radio-labelled probes were generated from the intron 3 region of *SBEIIb* (positions 2019 to 2391, see Figure 2) gene using the Megaprime DNA labelling system (Amersham Pharmacia Biotech UK Ltd) and used for hybridization under stringent conditions. Aus17340 and Aus10103 were missing the
 5 ~ 4.8 kB band from the D genome and Aus12509 and Aus12565 were missing the ~ 3.4 kB band from the B genome (Figure 10). These lines are therefore confirmed to be null mutants for the D or B genome *SBEIIb* genes, respectively.

Generation of B+D double null mutants.

10 The following crosses were carried out:

Aus17340a x Aus12509

Aus17340b x Aus12509

Aus17340a x Aus12565

Aus17340b x Aus12565

15 Aus12745 x Aus12509

Aus12745 x Aus12565

Aus17340a and Aus17340b are two different biotypes of the same Aus17340 cultivar; both were confirmed to be null for the D genome *SBEIIb* gene marker. The F1 plants
 20 were selfed and the F2 progeny screened by the PCR method for plants that were mutant in both the B and D genome *SBEIIb* genes (double null mutants). Segregation of the *SBEIIb* mutations was observed by PCR amplification using the primer pairs AR2b19cF (5'-CTATGCCAATTGAACAACAATGC-3') [SEQ ID No. 20] and AR2b23cR (5'-CGTGTTTCATCAATGTCTGAACG-3') [SEQ ID No. 21] (which
 25 amplifies the same region as ARA19F/ARA 23R) followed by digestion with the restriction enzyme *Rsa*I. A typical segregation pattern is shown in Figure 11. Chi square analysis revealed that the segregation pattern of the crosses Aus17340a x Aus12509 and Aus17340a x Aus12565 fitted in the expected ratio of 9:3:3:1 (Table 9). The segregation was highly distorted in the other crosses.

30

Table 9. Chi square analysis of F2 population of crosses between *SBEIIb* null mutants in the B and D subgenomes

Cross/ Phenotypes	17340a x 12509	17340b x 12509	1734a x 12565	17340 x 12565	12745 x 12509	12745 x 12565
Normal	85	63	56	72	95	21
B null	38	39	25	35	11	2

64

D null	23	29	11	11	57	16
BD null	6	10	4	6	3	0
Total	152	141	96	124	166	39
$\chi^2_{(9:3:3:1)}$	5.52	9.73	6.19	12.91	39.79	16.66

Tabled value of $\chi^2_{(9:3:3:1)}$ (0.05), df 3 = 7.81

- Albino plants were detected in all of the crosses irrespective of the parental lines, indicating that a mutant chlorophyll-related gene was also segregating in the populations. Of 24 albino plants analysed, 23 were B+D double null mutants and one appeared to be wild-type. Five normal looking green plants with B+D double null mutations were identified from 718 lines tested. Three of them were from the cross Aus17340b x Aus12509 (BD219, BD303, BD341), one from the cross Aus17340a x Aus12509 (BD54) and one from Aus17340b x Aus12565 (BD636). The results revealed that the mutations in the B and D genome *SBEIIb* genes were closely linked to a mutation in a chlorophyll-related gene which was giving the albino phenotype when two mutated loci came together. However, recombination events between the *SBEIIb* gene and the chlorophyll-related gene were identified, giving rise to normal B+D double null mutant lines, although at a very low frequency. This indicates that the two genes are closely linked but can be separated.

EXAMPLE 9. *SBEIIa* AND *SBEIIb* ARE LINKED IN WHEAT.

Isolation of BAC clones

- A large-insert cosmid binary cosmid (BAC) library constructed from *A. tauschii* variety *meyeri* (Mouillet et al., 1999) was probed with the intron 3 region of *SBEIIb* gene (positions 2019 to 2391, Figure 2) to isolate BACs containing the *SBEIIb* gene. Four positive clones were isolated and designated BAC-4, -5, -9 and -12. To confirm that they contained the *SBEIIb* gene, DNA from these clones was extracted, digested with *HindIII* or *EcoRI* and a Southern blot hybridisation carried out using the same probe (Figure 12). The clone BAC-5 showed one strong hybridising band of size ~7.5 kB with *EcoRI* and four bands of sizes ~6.1, 3.6, 2.3 and 1.7 with *HindIII* (Figure 12). This demonstrated the presence of *SBEIIb* on BAC-5. To test for the presence of the 3' region of the gene on BAC-5, PCR amplifications were carried out on this clone using specific primers designed to amplify exons 17 (AR2b3pr2F, 5'-GGATATGTATGATTTTCATGG-3' [SEQ ID No. 22], and AR2b3pr2R, 5'-CCATAAAGTTAAGATAACCC-3') [SEQ ID No. 23] and 20 (AR2b3pr1F, 5'-

GACATCAGACCACCAGTACG-3' [SEQ ID No. 24], and AR2b3pr1R, 5'-CTTCCCAGGCTTTAAACAGC-3') [SEQ ID No. 25], based on *SBEIIb* cDNA sequence. Both sets of primers amplified the expected products of sizes 128bp for exon 17 and 145bp for exon 20 indicating that BAC-5 contained the 3' end of *SBEIIb*. This
 5 was further confirmed by sequencing the PCR product from exon 20.

BAC-5 was also tested for the presence of the *SBEIIa* gene in addition to *SBEIIb*. Nucleotide sequencing reactions using the primer AR2akpn1F 5'-GGTACCGCAGAAAATATACGAGATTGACCC-3' [SEQ ID No. 26] yielded the
 10 sequence corresponding to the intron 3 region of the *SBEIIa* gene, being the same as the sequence from positions 2265 to 2478 (Figure 1) of *wSBE II-D1*. This result suggested that *SBEIIa* was also present on BAC-5, and meant that *SBEIIa* and *SBEIIb* were probably closely linked in wheat.

15 *Fluorescence in situ hybridization (FISH).*

In situ hybridization with the wSBE II-D1 genomic clone F2 (Rahman et al., 2001) and a wSBE II-D2 clone (Rahman et al., 2001) was performed on chromosome squashes from *Aegilops tauschii* and wheat as described by Turner et al., (1999). The identity of the hybridized chromosome was verified by double labeling with pSc119.2, a repetitive
 20 sequence used for chromosome identification (Mukai et al., 1990). Both of the wSBEII clones hybridized to the proximal region of chromosome 2 (Figure 13), indicating the proximity of the two *SBEII* genes in wheat.

Wheat SBEIIb null mutants are also mutant for SBEIIa

25 The *SBEIIb* null mutants identified as described above were screened for mutations in the *SBEIIa* gene using the primers Sr913F (5'-ATCACTTACCGAGAATGGG-3') [SEQ ID No. 27] and E6R (5'-CTGCATTGATTCCAATTG-3') [SEQ ID No. 28]. These primers were designed to amplify the intron 5 region of wSBE II-D1 and distinguish the *SBEIIa* genes on the A, B and D genomes.

30

The *SBEIIb* B genome null mutants Aus12565 and Aus12509 were found to be also B genome null mutants of the *SBEIIa* gene. Similarly, the D genome null mutants of *SBEIIb*, Aus17340 and Aus10103, were also D genome null mutants of *SBEIIa*. Furthermore, the B+D genome double mutant lines of *SBEIIb*, BD341 and BD636, were
 35 also B+D genome double null mutants of the *SBEIIa* gene. The data proves that *SBEIIa* and *SBEIIb* are closely linked in wheat, in contrast to rice and maize, and indicate that the

mutations for the B and D genome copies of these genes described above represent deletion mutations.

Triple null SBEIIa wheat mutants.

- 5 The methods described above may be used to isolate A genome mutants of *SBEIIa* and/or *SBEIIb*. For example, regions of BAC-5 closely linked to *SBEIIa* and/or *SBEIIb* are used as probes or for the design of PCR primers to screen for A-genome mutations in the genes. A-genome mutants are crossed with the B+D double null lines to produce a A+B+D triple null line. Alternatively, mutagenesis of the B+D genome double null
10 mutants is carried out by irradiation or other means and a triple null mutant entirely lacking *SBEIIa* activity and optionally *SBEIIb* activity is identified. A non-transgenic wheat variety with very high amylose levels is thereby provided.

EXAMPLE 10. MUTATION OF SBEIIA GENE IN WHEAT.

- 15 Mutation of the *SBEIIa* gene in wheat leading to reduced activity of *SBEIIa* can be achieved through either gamma ray irradiation or chemical mutagenesis, for example with ethyl methane sulfonate (EMS). For gamma ray induced mutation, seeds are irradiated at a dose of 20-50 kR from a ⁶⁰Co source (Zikiryaeva and Kasimov, 1972).
20 EMS mutagenesis is performed by treating the seeds with EMS (0.03%, v/v) as per Mullins et al., (1999). In a B+D double null background, mutant grains are identified on the basis of increased amylose content or altered starch grain morphology and confirmed by the methods described above. Mutants in *SBEIIa* that retain *SBEIIb* activity can be re-mutagenized and the progeny screened for loss of *SBEIIb* activity in
25 addition to *SBEIIa*, or the *SBEIIa* mutant can be crossed with an *SBEIIb* mutant to combine the mutations and produce a non-transgenic variety of wheat substantially lacking *SBEII* activity in the endosperm.

**EXAMPLE 11. SGP-1 WHEAT MUTANTS ARE REDUCED IN SBEIIa AND
30 SBEIIb ACTIVITY.**

- The genes for starch synthase II (SSII) from the A, B and D genomes of wheat (*Triticum aestivum*) encode polypeptides of 100-105 kDa that are also known as Starch Granule Proteins-1 (SGP-1). SSII (SGP-1) consists of three polypeptides of
35 approximate molecular masses of 100, 104 and 105 kDa which are encoded by a homeologous set of genes on the short arm of chromosomes 7B, 7A and 7D respectively (Denyer et al., 1995; Yamamori and Endo, 1996). Yamamori et al. (2000)

- produced an SGP-1 null wheat by crossing lines which were lacking the A, B and D genome specific forms of SGP-1 protein as assayed by protein electrophoresis. Examination of the SGP-1 null seeds showed that the mutation resulted in alterations in amylopectin structure, elevated amylose content and deformed starch granules (Yamamori et al., 2000). In addition, electrophoretic experiments on mature grain revealed that the levels of granule-bound SBEII (SGP-2) and SSI (SGP-3) decreased considerably. The molecular basis of the mutation(s) leading to the SGP-1 null line was not known.
- 10 We undertook experiments to further characterize a wheat line completely lacking SGP-1 in starch granules from mature grain. To determine whether the SSII genes are present in each of the A, B and D genomes of SGP-1 null wheat, DNA was extracted from the SGP-1 null wheat and wild-type (control) cv Chinese Spring wheat and analysed by PCR using the primer combinations ssIIa (5'-
- 15 CCAAGTACCAGTGGTGAACGC-3') [SEQ ID No. 29] and ssIIb (5'-CGGTGGGATCCAACGGCCC-3') [SEQ ID No. 30] for B genome or ssIIa and ssIIc (5'-CATGTGAGCTAGCTTTCGCCC-3') [SEQ ID No. 31] for the A and D genomes. The amplified region was between positions 2472-2821 bp of wSSIIA (GenBank accession no. AF155217) or the corresponding regions of wSSIIB or wSSIID. The
- 20 amplified region constituted a part of exon 8 and was chosen because it allowed the clear discrimination of the A, B and D genome products. Amplification was performed using 35 cycles at 94°C for 30 sec, 60°C for 1 min and 72°C for 2 min. The PCR fragments produced from A, B and D genomes of SGP-1 null wheat were the same size as the corresponding fragments produced from Chinese Spring. PCR amplification of gene
- 25 segments of the isoamylase and SSI genes, which are the most closely located starch biosynthetic genes to SSII and positioned on either side of SSII (Li et al., 2002), showed that these genes could be amplified from each of the A, B and D genomes of SGP-1 null wheat. Therefore, the SGP-1 null phenotype was not caused by the deletion of any of these genes on the short arm of chromosome 7.
- 30 When examined by scanning electron microscopy, starch granules from SGP-1 null developing seed, from 10 days post anthesis to maturity, were clearly deformed. The chain length distribution of debranched starch in the mutant showing an increase in the ratio of shorter chains (up to DP 8) and a decrease in the ratio of DP 9-22 when
- 35 examined by capillary electrophoresis.

Expression of starch synthases and branching enzymes in SGP-1 endosperm.

The expression of starch synthases and branching enzymes in the starch granules was investigated in the SGP-1 null and compared to those in wild-type cultivar Chinese Spring. Regardless of the stage of seed development, there was significant reduction of approximately 90%-96% in the amounts of SBEII and SSI in the granules in the SGP-1 null line, in addition to the absence of SSII (Figure 14). Use of specific antibodies showed that the SBEII band obtained from the granules was composed of SBEIIa and SBEIIb in the approximate ratio of 1:3 in Chinese Spring. In the SGP-1 null mutant, the amount was so low that the relative proportions could not be determined using the antibodies. There was also a decrease in the GBSS I level from an early stage of grain development. It is clear that in the SGP-1 mutant there is a reduced level of starch granule-associated polypeptides including SBEIIa and SBEIIb. The reduction in starch-granule associated polypeptides (SBEII and SSI) was not observed in the grains of the wheat lines used to produce the SGP-1 null (Yamamori *et al.*, 2000) and suggests that the effect is specifically caused by the absence of SSII.

Branching enzymes and starch synthases were also analysed in the soluble phase of developing endosperm. While the relative amount of soluble SBEIIb was similar in Chinese Spring and the SGP-1 null line, there was a reduction in the amount of SBEIIa in the soluble phase of the mutant (Figure 15). However, this may have been due in part to the genealogy of the SGP-1 null line.

These data demonstrate that SBEIIa activity can be decreased pleiotropically by mutation in the SSII gene. Although mutation in SSII alone led to relative amylose levels in the starch of less than 50%, it suggests that mutations in genes other than *SBEIIa* can be combined with *SBEII* mutations to increase amylose levels and produce altered starches.

EXAMPLE 12. KNOCKOUT OF SBEI –MULTIPLE ISOFORMS

Purification of wheat starch branching enzymes through anion exchange chromatography resolved three peaks of activity (Figure 16, Morell *et al.*, 1997). Endosperm extracts from the cultivar Chinese Spring (CS) revealed the presence of four SBEI polypeptides on a non denaturing PAGE using a polyclonal antibody, anti-WBE-1, raised against a synthetic peptide with the amino acid sequence corresponding to the N-terminal sequence of the protein of peak 1 (Figure 16B). Analysis of CS nullisomic-tetrasomic lines revealed that these polypeptides were encoded on chromosome 7; the bands on the immunoblot were assigned to the A (A band), B (B band) and D (Di and Dii bands) genomes and the activities were termed the A-major, B-major and D-major

activities, respectively. Immunoblot analysis of the purified fractions representing the active peaks obtained by anion exchange chromatography revealed that the first peak contained the SBEI A-major and D-major activities and the second peak contained the SBEI B-major activity (Figure 16C).

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The location of the gene encoding the major SBEI activity on chromosome 7 is consistent with the determined location of three well-characterised and related genes, wSBEI-D2, wSBEI-D3 and wSBEI-D4. The deduced protein sequence of SBEI-major showed that it is encoded by the last of these genes, wSBEI-D4 (Rahman et al., 1997, Suzuki et al., 2003). The presence of a fourth SBEI gene was suggested on the basis of Southern blot hybridization data (Suzuki et al., 2003).

10

Identification of null mutations of SBE I-major

In order to identify null mutations that lack expression of one or more SBEI isoforms, wheat germplasm collections were screened by immunoblot detection of SBEI-major after non-denaturing gel electrophoresis. The anti-wSBEI antibody described above was used. Of 182 Australian hexaploid wheat accessions analysed, 13 lines were identified that did not express SBEI-D major, 16 that lacked SBEI-B major, 10 that lacked SBEI-A, and two (Bindawarra and Vectis) that lacked both of the A and B isoforms. These lines were considered to have null mutations of the respective genome SBEI genes. The frequency of null mutations in the SBEI-major gene (~23%) was similar to that of the GBSS gene (22%) (Boggini et al., 2001).

20

Generation of SBE I-major triple null line

From the immunoblot analysis, it was clear that cultivars Bindwarra and Vectis were missing the A-major and B-major SBEI activities and the cultivar Cadoux was identified as missing the D-major activity. An F2 progeny population of 185 lines obtained from the cross Vectis x Cadoux were screened by immunoblotting. However, no lines missing all of the three activities were obtained, suggesting that either such progeny had low viability or there was some type of interaction between the genomes. Therefore, the progeny line VC3.1.11 that was missing the B- and D-major activities was crossed with a chromosome engineered Chinese Spring line (CS7AL-15) that was missing the A-major activity. Doubled haploid lines were screened by both PCR using the primers ARBE1CF (5'-GGGCAAACGGAATCTGATCC-3') [SEQ ID No. 32] and ARA9R (5'-CCAGATCGTATATCGGAAGGTCG-3') [SEQ ID No. 33] and immunoblotting and 2 lines (A113 and D13) out of 160 were that entirely lacked SBEI-major activity as

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judged by immunoblotting on non-denaturing gels. Figure 17 shows a representative segregation pattern of doubled haploid lines including A113 (lane 6).

Endosperm of line A113 was examined for residual SBE activity. A wild-type wheat
5 variety, D28, showed two peaks of SBEI activity. In contrast, A113 extracts gave the
first peak but the second peak of activity was completely missing. Amino acid
sequences obtained from the purified fraction comprising this activity indicated the
presence of a SBEI type of protein in A113. However, this fraction did not show a
reaction with anti-WBEI antibody in a non-denaturing gel. The branching activity in
10 A113 corresponded to a ~80 kDa protein which may be of a SBEII type enzyme as it
cross-reacted with potato SBE and maize SBEII antibodies.

These data demonstrate that *SBEI* mutant lines can be generated in wheat. Combination
of *SBEI* mutations with *SBEIIa* and optionally *SBEIIb* mutations produces wheat plants
15 having very high amylose levels in the grain starch.

**Example 13. Identification of mutant wheat lines comprising chromosome 2A
with a mutation in an *SBEII* gene.**

20 In an attempt to identify a wheat line having a mutation in an *SBEIIa* or *SBEIIb* gene,
2400 hexaploid wheat accessions were screened for null mutations of *SBEIIb* in the A, B
or D genomes. The primers AR2b19cF/AR2b23cR were used in PCR reactions on
genomic DNA samples of wheat plants of each line, followed by digestion of the
amplification products with *RsaI* and gel electrophoresis. This marker amplified the
25 intron 3 region (nucleotide positions 2085 to 2336 in wheat *SBEIIb* gene, Figure 2) and
was specific for *SBEIIb*. This screening had resulted in the identification of three D
genome *SBEII*-null mutants and two B genome *SBEII*-null mutants as described in the
Examples above. No mutant lines which lacked the A genome band corresponding to
SBEIIb were detected. This suggested that wheat lines comprising chromosome 2A with
30 a mutant *SBEIIb* gene do not occur naturally.

A gamma ray (⁶⁰Co source) induced mutant wheat population generated by Tony Prior
and Rohit Mago (CSIRO) was used to screen for induced mutations in wheat *SBEII*.
The wheat population was generated from the F2 progeny of a cross, Gabo 1BL.1RS x
35 Veery 3. A total of 2694 mutant seeds from this population were screened as described
above in PCR reactions with the primers AR2b19cF and AR2b23cR. Two seeds,
designated MLT2B8 and MLT2D1, that came from one plant, were identified that lacked

the *SBEIIb* A genome allele (Figure 18). No seeds in the population were identified to contain null mutations of *SBEIIb* in the B or D genomes.

As shown in the Examples above, *SBEIIa* and *SBEIIb* genes were closely linked in wheat
5 on the long arm of chromosome 2. Accordingly, we tested DNA from these seeds for the presence or absence of the A genome *SBEIIa* gene with PCR reactions using the primers Sr913F/E6R. These primers amplify the intron 5 region of wSBEII-D1 (nucleotide positions 2959 to 3189, Figure 1 [SEQ ID No. 1]). After amplification, the products were electrophoresed on a 5% sequencing gel (ABI Prism DNA sequencer).
10 Fluorescently labeled products were analysed using the software Genescan. The scan profiles showed that the amplification products for both of the mutant seeds MLT2B8 and MLT2D1 lacked the product corresponding to the A genome *SBEIIa* gene, indicating that both seeds had null alleles for the A genome *SBEIIa* in addition to *SBEIIb*.

15 The null mutations in these seeds were further confirmed by using an A genome specific marker for *SBEIIa*, ARIIaAF (5'-GCAAAAGCCAGATCATAAATTAGAGC-3') [SEQ ID No. 34] and ARIIaAR (5'-CTTCCAATTCATTGTTAATGGTCACAC-3') [SEQ ID No. 35] that amplify only the product from A genome *SBEIIa* gene (nucleotide
20 positions 3024 to 3131 of wSBE II-DA1, Figure 1). While this pair of primers amplified a 110bp product from plant material from the variety Chinese Spring, this product was clearly missing in the two putative mutant seeds. This was the same as for the negative control dt2AS, which is a chromosome engineered line of Chinese Spring that is missing the long arm of chromosome 2A. Since the *SBEIIa* and *SBEIIb* genes are
25 located on the long arm of chromosome 2, this line lacks the A genome allele of both these genes and hence could be used as a negative control (Figure 19).

The embryos from the mutant seeds MLT2B8 and MLT2D1, identified to be A genome mutants for *SBEIIa* and *SBEIIb*, are grown to produce plants. The starch obtained from
30 seed from these plants is analysed for amylose content, chain length and other properties to determine if the null mutations of both the *SBEIIa* and *SBEIIb* on the A genome affect starch properties.

As described above, five lines having mutation in both the B and D genome *SBEIIa* and
35 *SBEIIb* genes had been generated. Of these, lines BD 219 and BD 636 are grown in a greenhouse and crossed to the A null mutant lines MLT2B8 and MLT2D1. A doubled haploid population is generated from the F1 seeds of these crosses to provide

homozygous triple null mutant plants. Such triple null mutant plants should occur in doubled haploid populations at a frequency of 1 in 8. The A genome null mutations can be combined with either the B genome mutations or the D genome mutations by similar crosses. In further crosses, any of the null alleles can be introduced into any suitable
 5 genetic background for agronomic or other traits.

The following crosses are performed to produce durum wheat (such as, for example, variety Wollaroi) having mutations in the A genome *SBEIIa* and *SBEIIb*:

- 10 1) Wollaroi x MLT2B8 or MLT2D1, to produce A-genome *SBEIIa/SBEIIb* null durum in Wollaroi background.
- 2) A-genome null durum (Wollaroi) x B-genome null *SBEIIa/SBEIIb* wheat line to produce AB double null *SBEIIa/SBEIIb* durum (Wollaroi)

Alternatively,

- 15 1) Wollaroi x B-genome null wheat line, to produce B-genome null durum (Wollaroi)
- 2) B-genome null Wollaroi x A null wheat line, to produce AB double null *SBEIIa/SBEIIb* durum.

20 These crosses result in the generation of high amylose durum wheat which will have specific end uses with health benefits similar to that of high amylose hexaploid wheat.

Example 14. Confirmation of the high amylose content in grain by Sepharose 2B column separation methods

25

The amylose content of starch in the grain of transgenic wheat plants containing *SBEIIa/SBEIIb* inhibitory genetic constructs was determined by a Sepharose column separation method. In this method, starch molecules were separated on the column based on their molecular weight. The separated fractions were then assayed using the Starch
 30 Assay Kit (Sigma) according to the suppliers instructions.

Approximately 10mg of starch was dissolved in 3.0 ml of 1N NaOH (de-gased) by incubation at 37°C for 30 min. The starch solution was centrifuged for 15 min to spin down the undissolved components. The supernatant was loaded on to a Sepharose
 35 CL2B column at a pump speed of 1ml/min. The column was run using 10mM NaOH as buffer and fifty fractions of 2.5 ml each were collected. The pH of fractions 9 to 50 was

adjusted to 4.5 with 35 μ l of 1 M HCl. An aliquot (250 μ l) of each sample was transferred into a tube followed by the addition of 250 μ l of Starch reagent (Starch assay kit, Sigma). The controls included: a starch assay reagent blank containing only starch reagent (250 μ l) and water (250 μ l), a glucose assay reagent blank containing only 500 μ l water, a sample blank containing only 250 μ l starch sample and 250 μ l water and a sample test containing only 250 μ l starch reagent and 250 μ l starch sample. The samples and the controls were incubated at 60°C for 60 min, and then 200 μ l of each transferred to a new tube followed by addition of 1 ml of glucose reagent (starch assay kit, Sigma) and incubation at 37°C for 30 min. The absorbance at 340 nm was used to determine the quantity of starch (mg) in each fraction according to the instructions supplied with the kit.

The chromatogram of starch samples revealed two peaks eluted from the Sepharose column. The amylose content (second peak) of each sample was calculated as a percentage of the total amount of starch within both of the peaks.

Using this method, the amylose content of the ds-SBEIIa transgenic line Acc. 144087, which was shown to be homozygous for the transgene, was calculated to be 78% and that of a ds-SBEIIb transgenic line Acc 144008 (homozygous transgenic line from the event IIb 110.16b) was estimated to be 23% (Figure 20). In comparison, the iodometric method gave amylose contents for these lines of 88.47% and 27.29%, respectively (Table 10).

Functional properties such as gelatinization temperature, paste viscosity and starch swelling volume are analysed by Differential Scanning Calorimetry (DSC), Rapid Visco Analyser (RVA) and starch swelling power test, respectively. The structure of these starches is analysed by X-ray crystallography and particle size analysis.

Table 10. Amylose content of wheat transgenic lines estimated by iodometric method

Line	Target enzyme	Event No.	Amylose content (%)
NB1	Non transformed	-	31.8
144008	SBE IIb	IIb 110.16b	27.3
144087	SBE IIa	IIa 85.3a	88.5
144025	SBE IIa	IIa 50.1b	75.8
LSD	-	-	7.7

5

Example 15. Chain length distribution analysis.

The chain length distribution of starch samples was determined by fluorophore assisted carbohydrate electrophoresis (FACE) after isoamylase de-branching of the starch. The percentages of chain lengths from DP 6-11, DP 12-30 and DP 31-60 in starch from the transgenic seed compared to non-transgenic controls are presented in Table 11. Molar difference plots in which the normalized chain length distributions for starch from high amylose transgenic lines were subtracted from the normalized distribution for starch from the isogenic non transformed controls are given in Figure 21.

15

Table 11. Chain length distribution of isoamylase debranched starches from wheat transgenic lines

Line	Targeted gene	Event No	DP4-12	DP13-24	DP24-36	>36
NB1	Nontransformed control	-	57.39	37.38	3.83	1.40
144087	SBEIIa	IIa 85.3a	47.40	42.27	6.16	4.17
144025	SBEIIa	IIa 50.1b	49.99	44.40	5.60	-
144008	SBEIIb	IIb 110.16b	57.98	37.65	4.37	-

20 There was a significantly lower proportion of chain lengths of DP 4-12 in starch from ds-SBEIIa transgenic seed compared to starch from untransformed seed or ds-SBEIIb transgenic seed. The proportion of chain lengths of >DP 13 was higher in ds-SBEIIa transgenic seed compared to the others. These results suggest the possibility that

SBEIIa is selectively involved in the synthesis of shorter chains of DP 4-12 in wheat starch. In starch from the SSIa mutant, however, there was an increase in the proportion of shorter chain lengths in the amylose.

5 Example 16. Properties of starch from transgenic wheat.

Physical properties of starch from ds-SBEIIa and ds-SBEIIb transgenic lines including the gelatinisation temperature were analysed using a Perkin Elmer Diamond differential scanning calorimeter. Approximately 20mg of each starch was mixed with water at a ratio of 1:2 i.e. to a moisture content of 66.7%, and sealed in a DSC pan. A heating rate of 10°C per minute was used to heat the test and reference samples from 0 to 150°C. Data were analysed using the software available with the instrument.

Two endotherm peaks were observed in the thermogram DSC trace for each starch. The first peak represented the breakdown of crystalline structure during gelatinization of

Lines	Enzyme targeted	Peak 1 (Gelatinisation)					Peak 2 (Amylose-lipid dissociation)			
		Onset	Peak	End	Area	ΔH	Onset	Peak	End	ΔH
008	SBE IIb	58.8	63.7	70.8	234.8	4.5	93.2	103.5	110.3	0.7
012	SBE IIb	59.0	64.1	70.8	262.6	4.3	94.5	103.1	109.7	0.6
121	SBE IIa	53.7	67.5	86.9	156.4	2.6	92.4	102.9	108.9	0.7
087	SBE IIa	53.1	71.9	85.9	142.6	2.4	95.7	102.7	108.9	0.7
114	SBE IIa	53.0	68.1	88.0	125.2	2.1	92.8	102.5	109.6	0.8
109c	Control *	55.9	60.7	68.8	234.3	3.9	97.2	104.6	109.9	0.4

starch. The second peak represented the amylose-lipid dissociation endotherm. The gelatinization peak temperature of starch from ds-SBEIIa transgenic lines showed an increase of approximately 7-10°C compared to the peak temperature for a non-transformed control starch, and approximately 3 to 7°C increased compared to starch from a ds-SBEIIb transgenic line.

Table 12. Thermal properties of transgenic wheat starch measured by differential scanning calorimeter (DSC).

A marked increase in the end temperature of gelatinization (first peak) of approximately 16-19°C was observed in these lines compared to both non-transformed control and ds-SBEIIb transgenic lines. The temperature of onset of gelatinization appeared to be earlier in ds-SBEIIa transgenic lines than the control or ds-SBEIIb transgenic lines. Ng et al., 1997 reported a gelatinization onset temperature of *amylose extender (ae)* maize starch

similar to that of normal maize starch, but a significant increase in the peak gelatinization temperature in *ae* starch compared to normal starch. The gelatinization enthalpy of starch from ds-SBEIIa transgenic lines was significantly lower than that of both the control and ds-SBEIIb lines. This seems to be reflecting the significantly lower gelatinization peak area which represents the reduced amount of amylopectin in ds-SBEIIa transgenic lines. No significant alteration was observed in the amylose-lipid dissociation peak in any of the transgenic lines. We have therefore obtained starch with this novel set of properties.

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CLAIMS

1. Grain obtained from a wheat plant, comprising starch, wherein the proportion of amylose in the starch of the grain is at least 50%.
5
2. The grain of claim 1, comprising a reduced level of SBEIIa protein, SBEIIa enzyme activity or both in the endosperm relative to wild-type grain.
- 10 3. The grain of either claim 1 or 2, comprising a genetic variation which leads to a reduction in the level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain, which genetic variation comprises a mutation of an SBEIIa gene or an introduced nucleic acid which encodes an inhibitor of SBEIIa gene expression.
15
4. Grain obtained from a wheat plant, the grain comprising starch and a genetic variation which leads to a reduction in the level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain, which genetic variation comprises a mutation of an SBEIIa gene or an introduced nucleic acid which
20 encodes an inhibitor of SBEIIa gene expression;
wherein the proportion of amylose in the starch of the grain is at least 30%.
5. The grain of claim 4, wherein the genetic variation comprises a mutation in an SBEIIa gene which leads to a reduced level of SBEIIa protein, SBEIIa enzyme activity
25 or both in the endosperm relative to wild-type grain.
6. The grain of any one of claims 1 to 5, comprising a null mutation in at least one SBEIIa gene.
- 30 7. The grain of claim 6, wherein the plant is hexaploid and the grain comprises null mutations in two or three SBEIIa genes.
8. The grain of any one of claims 1, 2 or 4 comprising a transgene which encodes an inhibitor of SBEIIa gene expression.
35
9. The grain of claim 8, wherein the transgene encodes an antisense, co-suppression, ribozyme or duplex RNA molecule.

10. The grain of any one of claims 1 to 9, further comprising a reduced level of SBEIIb protein, enzyme activity or both relative to wild-type grain.
- 5 11. The grain of any one of claims 1 to 10, comprising a reduced level of SBEI protein, enzyme activity or both relative to wild-type grain.
12. The grain of any one of claims 1 to 11, comprising an altered level of an enzyme relative to wild-type grain, wherein said enzyme is selected from the group consisting of
10 ADP glucose pyrophosphorylase, GBSS, SSI, SSII, SSIII, a debranching enzyme of an isoamylase type, a debranching enzyme of a pullulanase type and any combination of these.
13. The grain of either claims 4 or 5, wherein the proportion of amylose in the starch of
15 the grain is at least 40%.
14. The grain of any one of claims 1 to 13 which is non-shrunken or which has an average weight of at least about 36 mg or both.
- 20 15. The grain of any one of claims 1 to 14 wherein at least 50% of starch granules from the grain appear non-birefringent when observed under polarized light.
16. The grain of any one of claims 1 to 15, wherein the starch content of the grain when naked is at least 25% (w/w) or which has a starch content that is at least 90% of
25 the starch content of wild-type grain or both.
17. The grain of any one of claims 1 to 16 which is whole grain or milled, ground, pearled, rolled, kibbled, par-boiled or cracked grain.
- 30 18. A wheat plant capable of producing the grain of any one of claims 1 to 17.
19. Starch granules extracted from the grain of any one of claims 1 to 17.
20. The starch granules of claim 19 which comprise starch, wherein the proportion of
35 amylose in the starch of the granules is at least 50%.
21. Starch extracted from the grain of any one of claims 1 to 17.

22. A product comprising the grain of any one of claims 1 to 17, or flour, wholemeal, semolina or starch produced from the grain of any one of claims 1 to 17, or any combination of these.
- 5 23. The product of claim 22 wherein the flour, wholemeal, semolina or starch is blended with flour, wholemeal, semolina or starch from another source.
24. The product of claim 22 which is a non-food product.
- 10 25. A composition comprising the starch of claim 21 and another food ingredient or water.
- 15 26. A method of producing a wheat plant capable of producing grain comprising the steps of
- i) introducing a genetic variation into a parent wheat plant or seed; and
 - ii) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to a wild-type plant or seed;
- 20 wherein the grain comprises starch, wherein the proportion of amylose in the starch of the grain is at least 50%.
27. A method of producing a wheat plant capable of producing grain comprising the steps of
- 25 i) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a mutation of an SBEIIa gene or an introduced nucleic acid which encodes an inhibitor of SBEIIa gene expression, and
- 30 ii) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain;
- wherein the grain comprises starch and wherein the proportion of amylose in the starch of the grain is at least 30%.
- 35 28. The method of claims 26 or 27 wherein the step of introducing the genetic variation comprises introducing an exogenous nucleic acid which expresses an inhibitor of SBEIIa gene expression.

29. The method of any one of claims 26 or 27 wherein the step of introducing the genetic variation comprises mutagenesis of the parent wheat plant or seed with a chemical agent or radiation.

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30. A method of producing a wheat plant capable of producing grain comprising the steps of

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- i) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a mutation of an SBEIIa gene, and
- ii) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both relative to wild-type grain;
- iii) introducing a genetic variation into a parent wheat plant or seed, wherein the genetic variation comprises a mutation of an SBEIIb gene, and
- 15 iv) identifying a progeny plant or seed of the parent wheat plant or seed which has a reduced level of SBEIIb gene expression, SBEIIb enzyme activity in the endosperm or both relative to wild-type grain;
- v) crossing a plant having a reduced level of SBEIIa gene expression, SBEIIa enzyme activity in the endosperm or both, with a plant having a reduced level of SBEIIb gene expression, SBEIIb enzyme activity in the endosperm or both;

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and identifying a wheat plant having reduced gene expression, enzyme activity or both, of both SBEIIa and SBEIIb.

25 31. A method of producing altered wheat starch comprising the step of extracting starch from the grain of any one of claims 1 to 17.

32. The wheat plant of claim 18 which is *Triticum aestivum* ssp. *aestivum*.

30 33. A method of identifying a wheat plant or seed having a mutation in an SBEIIa gene or an SBEIIb gene, comprising the steps of screening a population of wheat plants or seed with a molecular marker that is linked to the SBEIIb gene, or SBEIIa gene, respectively, of wheat; and identifying the plant or seed on the basis of the presence or absence of the linked molecular marker.

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34. A method of identifying a wheat plant or seed having a mutation in an SBEIIa gene, or an SBEIIb gene, comprising the steps of screening a population of wheat plants

or seed with an antibody that is specific for SBEIIb protein, or SBEIIa protein, respectively, of wheat; and identifying the plant or seed on the basis of the presence or absence of antibody binding.

5 35. Grain obtained from a wheat plant, comprising a mutation wherein the SBEIIa gene is absent from the long arm of chromosome 2A or wherein the SBEIIa gene on the long arm of chromosome 2A comprises a mutation which leads to reduced SBEIIa protein, SBEIIa enzyme activity, or both, in the endosperm of said grain relative to wild-type grain.

10 36. The grain of claim 35, wherein the mutation is a null mutation of the SBEIIa gene.

15 37. The grain of either claim 35 or 36, wherein the mutation is a deletion of at least part of the SBEIIa gene.

20 38. The grain of any one of claims 35 to 37, further comprising a mutation wherein the SBEIIb gene is absent from the long arm of chromosome 2A or wherein the SBEIIb gene on the long arm of chromosome 2A comprises a mutation which leads to reduced SBEIIb protein, SBEIIb enzyme activity, or both, in the endosperm of said grain relative to wild-type grain.

25 39. The grain of any one of claims 35 to 38, wherein the deletion disrupts expression of both the SBEIIa and SBEIIb genes on the long arm of chromosome 2A.

40. The grain of any one of claims 35 to 39, wherein the plant is a durum wheat plant.

30 41. The grain of claim 40, further comprising a genetic variation which leads to reduced starch branching enzyme activity encoded by the SBEIIa gene on the long arm of chromosome 2B relative to wild-type grain.

35 42. The grain of claim 41, wherein said genetic variation comprises an absence of the SBEIIa gene from the long arm of chromosome 2B or a mutation of the SBEIIa gene of the long arm of chromosome 2B which leads to reduced SBEIIa enzyme activity in the endosperm of said grain relative to wild-type grain.

43. The grain of any one of claims 35 to 39, wherein the plant is *Triticum aestivum* ssp *aestivum*.
44. The grain of claim 43, comprising a genetic variation which leads to reduced starch branching enzyme activity encoded by the SBEIIa gene on the long arm(s) of chromosome 2B, chromosome 2D or both chromosomes relative to wild-type grain.
45. The grain of claim 44, wherein said genetic variation comprises an absence of the SBEIIa gene from at least one of said chromosomes or a mutation of the SBEIIa gene of at least one of said chromosomes which leads to reduced SBEIIa enzyme activity in the endosperm of said grain relative to wild-type grain.
46. The grain of any one of claims 41 or 44, wherein said genetic variation comprises an introduced nucleic acid which encodes an inhibitor of SBEIIa gene expression, activity or both.
47. The grain of any one of claims 35 to 46, wherein the level of SBEIIa enzyme activity is reduced by at least 40% relative to wild-type grain.
48. The grain of any one of claims 35 to 47, wherein the proportion of amylose in the starch of the grain is at least 30%.
49. The grain of claim 48, wherein the proportion of amylose in the starch of the grain is at least 50%.
50. The grain of any one of claims 1 to 17, 35 to 49, wherein the amylopectin of the grain has a reduced proportion of the 4 - 12 dp chain length fraction relative to the amylopectin of wild-type grain, as measured after isoamylase debranching of the amylopectin.
51. The grain of any one of claims 35 to 50 which is non-shrunken.
52. The grain of any one of claims 35 to 51 which has an average weight of at least about 36 mg.
53. The grain of any one of claims 48 to 52, wherein at least 50% of starch granules from the grain appear non-birefringent when observed under polarized light.

54. The grain of any one of claims 46 to 53, wherein the starch content of the grain when naked is at least 25% (w/w) or which has a starch content that is at least 90% of the starch content of wild-type grain.
55. The grain of any one of claims 35 to 54 which is whole grain or milled, ground, pearled, rolled, kibbled, par-boiled or cracked grain.
56. The grain of any one of claims 35 to 55, further comprising a reduced level of SBEI protein, SBEI enzyme activity or both.
57. The grain of any one of claims 35 to 56, further comprising an altered level of an enzyme relative to wild-type grain, wherein said enzyme is selected from the group consisting of ADP glucose pyrophosphorylase, GBSS, SSI, SSII, SSIII, a debranching enzyme of an isoamylase type, a debranching enzyme of a pullulanase type and any combination of these.
58. A wheat plant capable of producing the grain of any one of claims 35 to 57.
59. Starch granules extracted from the grain of any one of claims 35 to 57.
60. The starch granules of claim 59 which comprise starch, wherein the proportion of amylose in the starch of the granules is at least 50%.
61. Starch extracted from the grain of any one of claims 35 to 57.
62. A product comprising the grain of any one of claims 35 to 57 or flour, wholemeal, semolina or starch produced from the grain of any one of claims 35 to 57.
63. The product of claim 62 wherein the flour, wholemeal, semolina or starch is blended with flour, wholemeal, semolina or starch from another source.
64. The product of claim 62 which is a non-food product.
65. A composition comprising the starch of claim 61 and another food ingredient or water.

66. A method of producing grain, comprising (i) growing a wheat plant, and (ii) harvesting grain from said wheat plant, said grain comprising a mutation wherein the SBEIIa gene is absent from the long arm of chromosome 2A or wherein the SBEIIa gene on the long arm of chromosome 2A comprises a mutation which leads to reduced SBEIIa enzyme activity in the endosperm of said grain relative to wild-type grain.
67. A method of producing starch, comprising (i) obtaining grain according to any one of claims 35 to 57, and (ii) extracting starch from said grain.
68. A method of producing the grain of any one of claims 1 to 17, 35 to 57, comprising (i) growing a wheat plant, and (ii) harvesting grain from said wheat plant.

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FIGURE 1

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FIGURE 1

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FIGURE 1

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11451

FIGURE 1

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FIGURE 2

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FIGURE 2

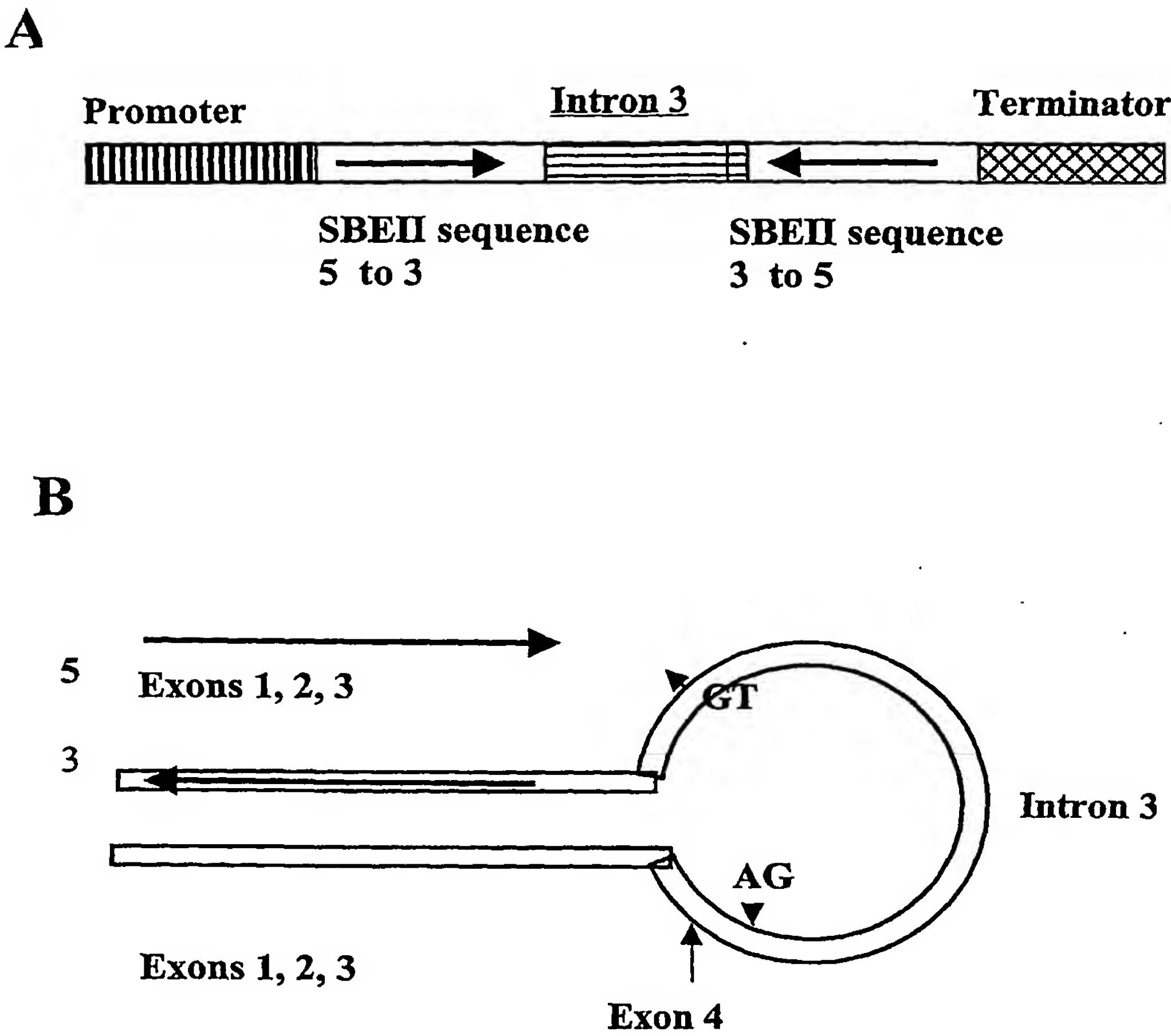
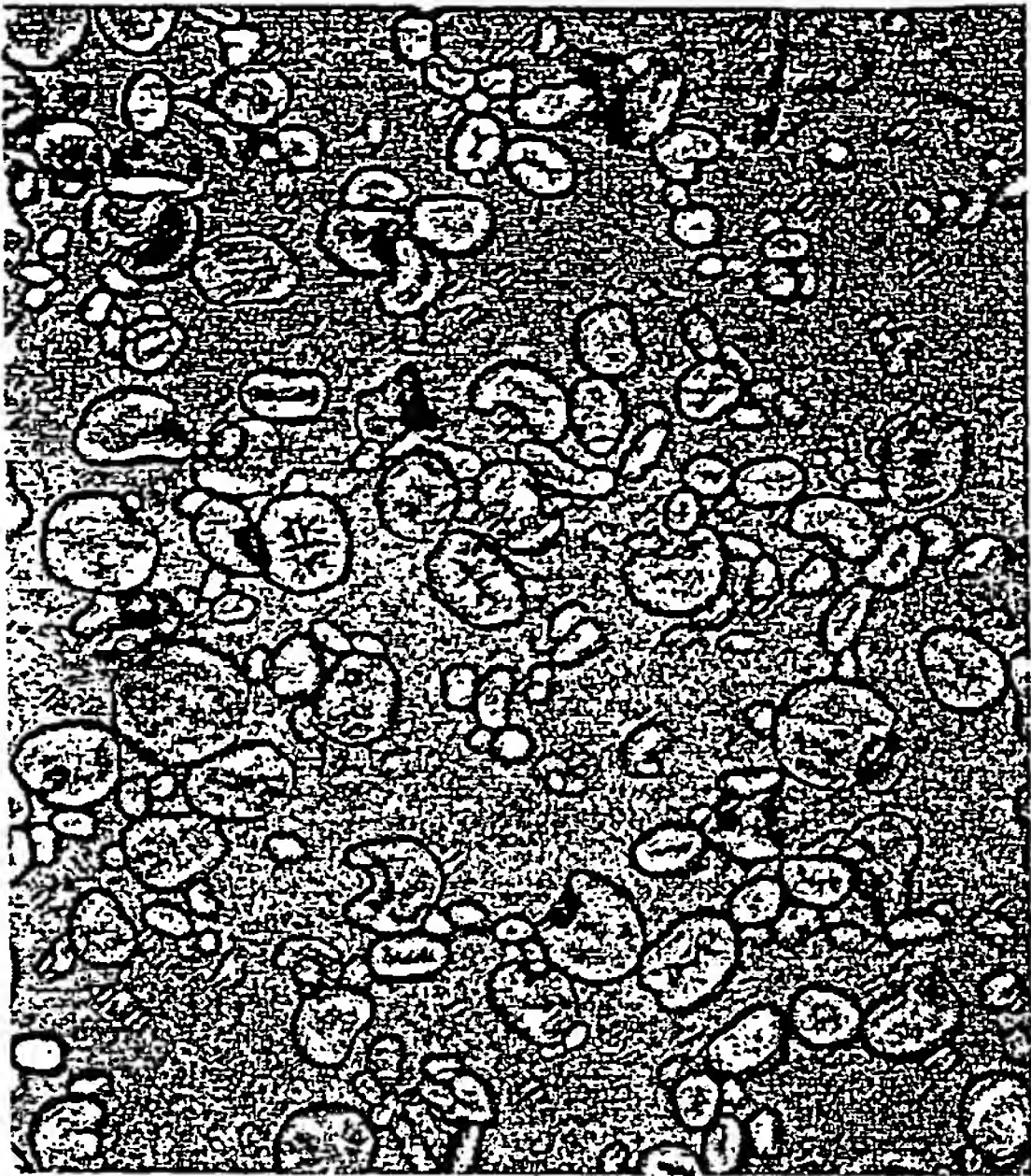


FIGURE 3

B



A

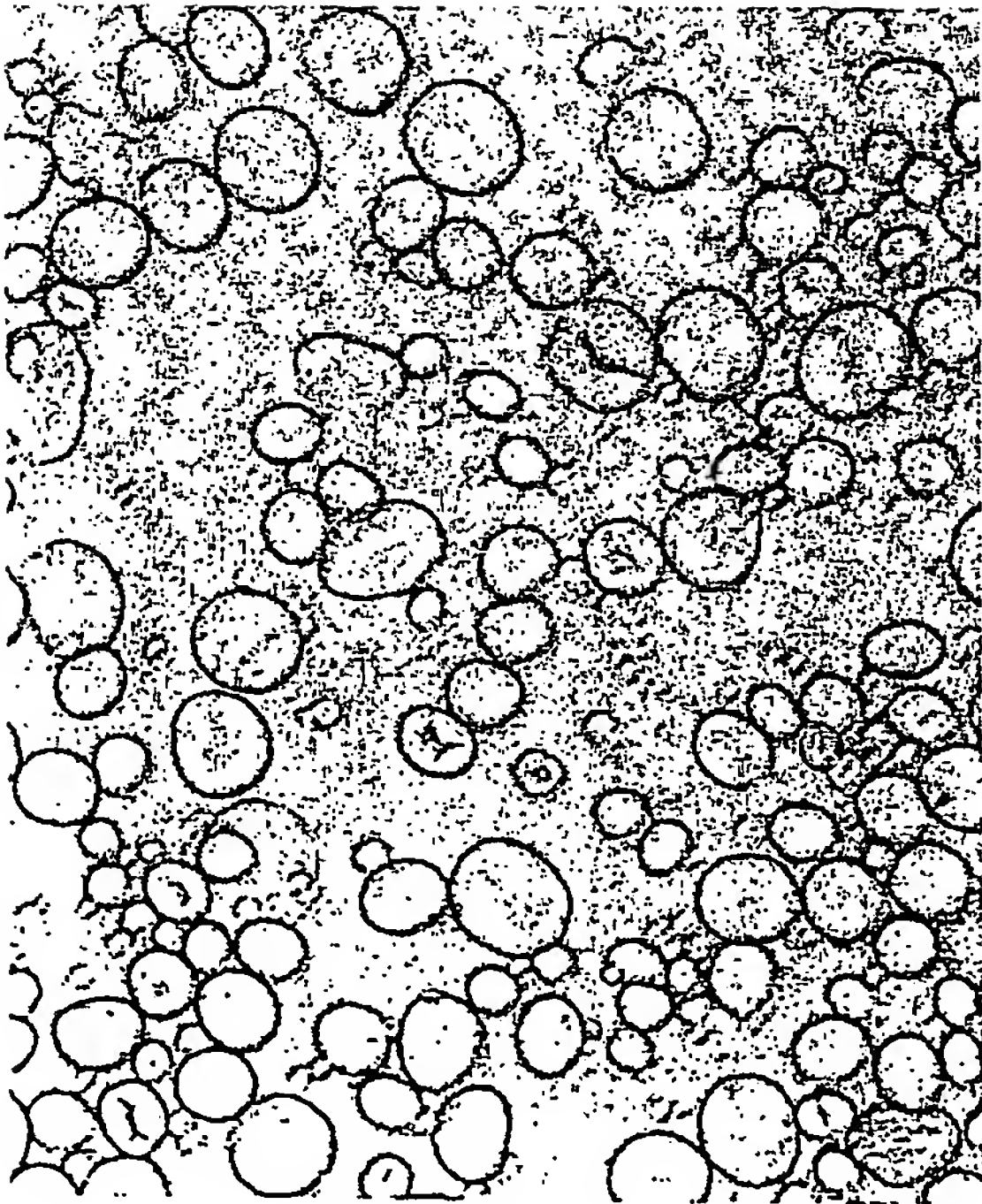
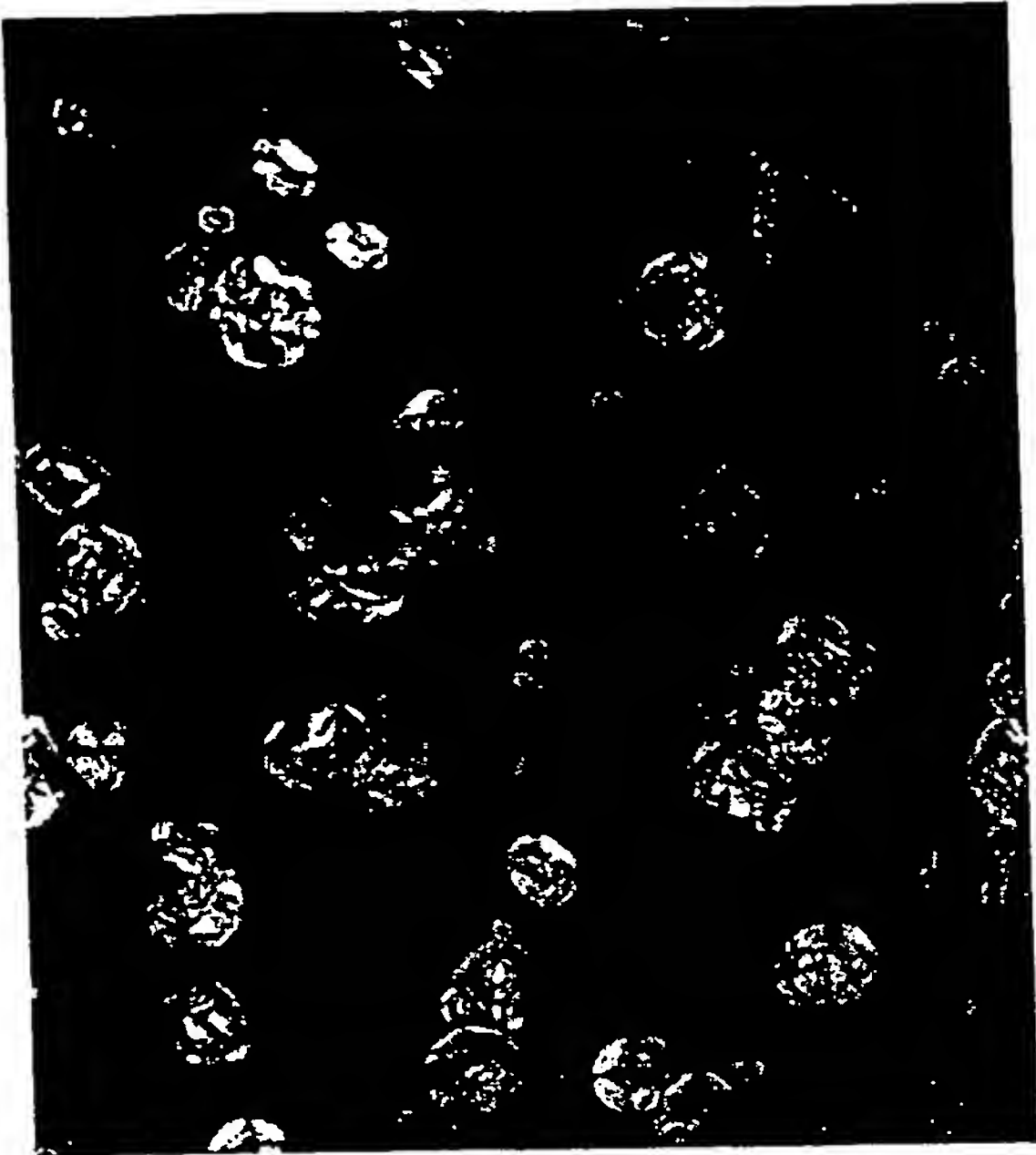


Figure 4

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B



A

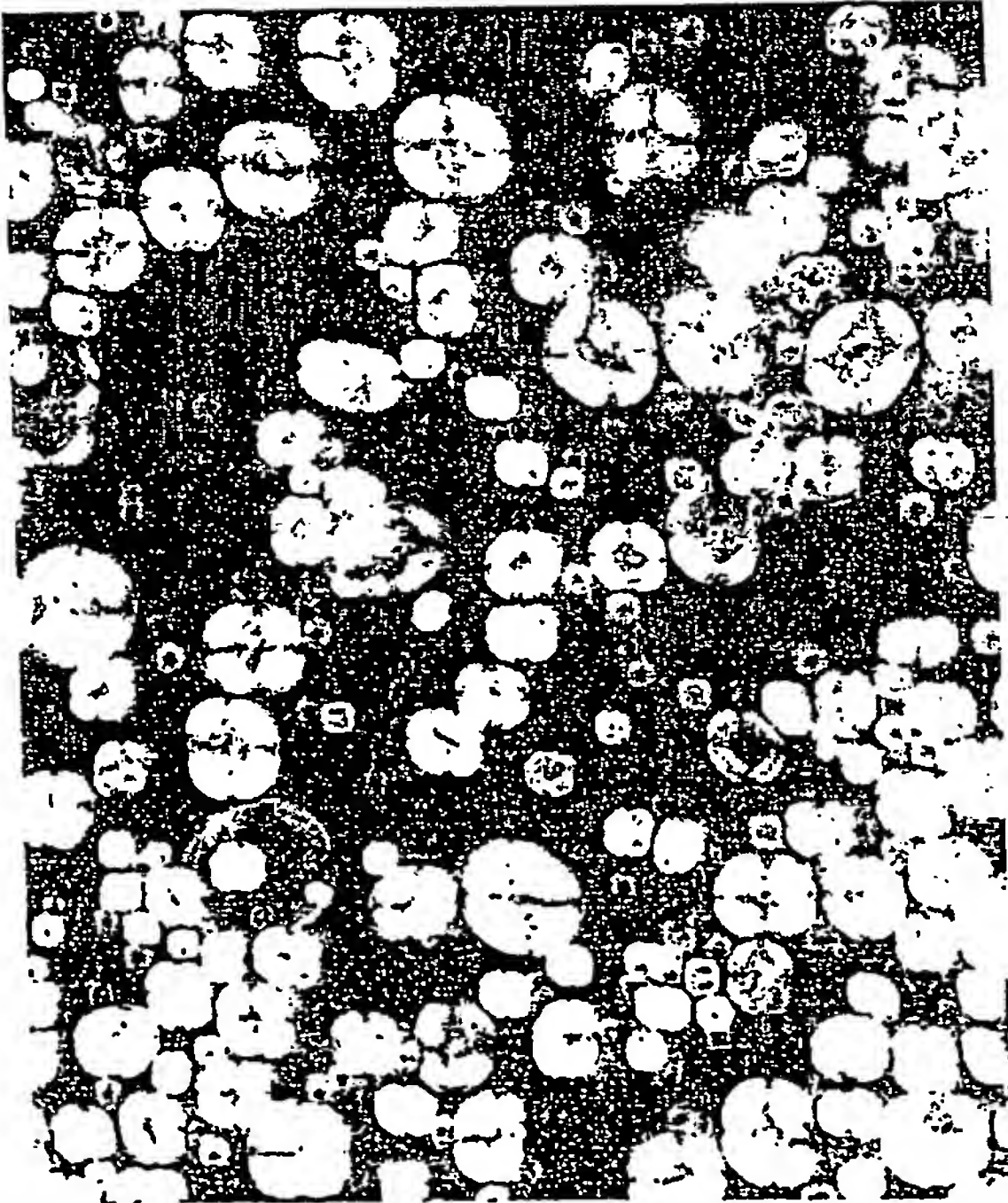


Figure 5

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FIGURE 6

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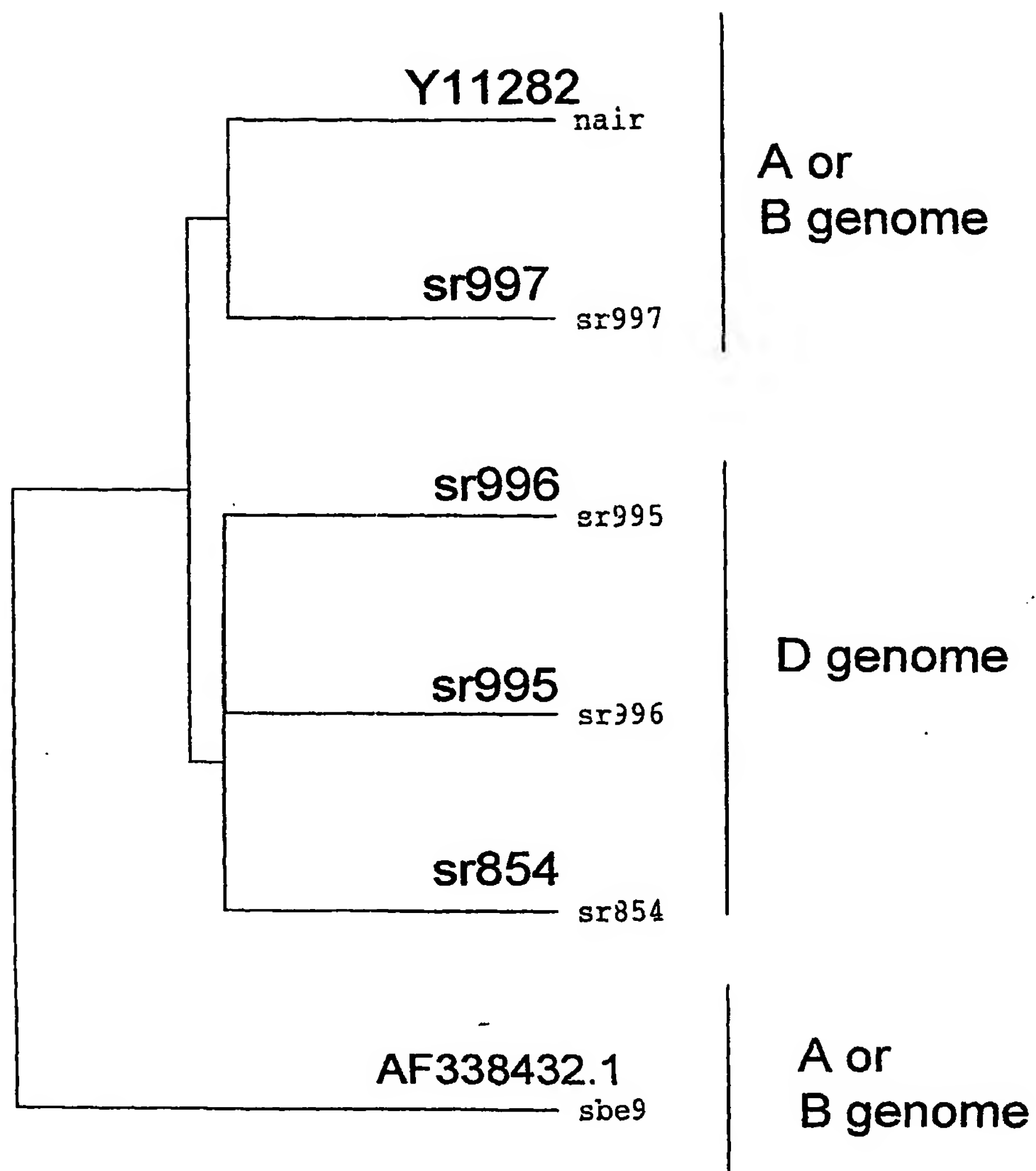


Figure 7

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sr854

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				20					25					30
Ala	Asp	Leu	Pro	Ser	Leu	Leu	Leu	Arg	Lys	Lys	Asp	Ser	Ser	Arg
				35					40					45
Ala	Val	Leu	Ser	Arg	Ala	Ala	Ser	Pro	Gly	Lys	Val	Leu	Val	Pro
				50					55					60
Asp	Gly	Glu	Ser	Asp	Asp	Leu	Ala	Ser	Pro	Ala	Gln	Pro	Glu	Glu
				65					70					75
Leu	Gln	Ile	Pro	Glu	Asp	Ile	Glu	Glu	Gln	Thr	Ala	Glu	Val	Asn
				80					85					90
Met	Thr	Gly	Gly	Thr	Ala	Glu	Lys	Leu	Gln	Ser	Ser	Glu	Pro	Thr
				95					100					105
Gln	Gly	Ile	Val	Glu	Thr	Ile	Thr	Asp	Gly	Val	Thr	Lys	Gly	Val
				110					115					120
Lys	Glu	Leu	Val	Val	Gly	Glu	Lys	Pro	Arg	Val	Val	Pro	Lys	Pro
				125					130					135
Gly	Asp	Gly	Gln	Lys	Ile	Tyr	Glu	Ile	Asp	Pro	Thr	Leu	Lys	Asp
				140					145					150
Phe	Arg	Ser	His	Leu	Asp	Tyr	Arg	Tyr	Ser	Glu	Tyr	Lys	Arg	Ile
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Arg	Ala	Ala	Ile	Asp	Gln	His	Glu	Gly	Gly	Leu	Glu	Ala	Phe	Ser
				165					170					175
Arg	Gly	Tyr	Glu	Lys	Leu	Gly	Phe	Thr	Arg	Ser	Ala	Glu	Gly	Ile
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Thr	Tyr	Arg	Glu	Trp	Ala	Pro	Gly	Ala	His	Ser	Ala	Ala	Leu	Val
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Gly	Asp	Phe	Asn	Asn	Trp	Asn	Pro	Asn	Ala	Asp	Thr	Met	Thr	Arg
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Asp	Asp	Tyr	Gly	Val	Trp	Glu	Ile	Phe	Leu	Pro	Asn	Asn	Ala	Asp
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Lys	Arg	Pro	Glu	Ser	Leu	Arg	Ile	Tyr	Glu	Ser	His	Ile	Gly	Met
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Ser	Ser	Pro	Glu	Pro	Lys	Ile	Asn	Ser	Tyr	Ala	Asn	Phe	Arg	Asp
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Glu	Val	Leu	Pro	Arg	Ile	Lys	Arg	Leu	Gly	Tyr	Asn	Ala	Val	Gln
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His	Val	Thr	Asn	Phe	Phe	Ala	Pro	Ser	Ser	Arg	Phe	Gly	Thr	Pro
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Glu	Asp	Leu	Lys	Ser	Leu	Ile	Asp	Arg	Ala	His	Glu	Leu	Gly	Leu
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Leu	Val	Leu	Met	Asp	Ile	Val	His	Ser	His	Ser	Ser	Asn	Asn	Thr
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Leu	Asp	Gly	Leu	Asn	Gly	Phe	Asp	Gly	Thr	Asp	Thr	His	Tyr	Phe
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His	Gly	Gly	Pro	Arg	Gly	His	His	Trp	Met	Trp	Asp	Ser	Arg	Leu

FIGURE 8

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Ala Arg Trp Trp Leu Glu Glu Tyr Lys Phe Asp Gly Phe Arg Phe			
	445	450	455
Asp Gly Val Thr Ser Met Met Tyr Thr His His Gly Leu Gln Met			
	460	465	470
Thr Phe Thr Gly Asn Tyr Gly Glu Tyr Phe Gly Phe Ala Thr Asp			
	475	480	485
Val Asp Ala Val Val Tyr Leu Met Leu Val Asn Asp Leu Ile His			
	490	495	500
Gly Leu Tyr Pro Asp Ala Val Ser Ile Gly Glu Asp Val Ser Gly			
	505	510	515
Met Pro Thr Phe Cys Ile Pro Val Pro Asp Gly Gly Val Gly Phe			
	520	525	530
Asp Tyr Arg Leu His Met Ala Val Ala Asp Lys Trp Ile Glu Leu			
	535	540	545
Leu Lys Gln Ser Asp Glu Ser Trp Lys Met Gly Asp Ile Val His			
	550	555	560
Thr Leu Thr Asn Arg Arg Trp Leu Glu Lys Cys Val Thr Tyr Ala			
	565	570	575
Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile Ala Phe			
	580	585	590
Trp Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp Arg			
	595	600	605
Pro Ser Thr Leu Arg Ile Asp Arg Gly Ile Ala Leu His Lys Met			
	610	615	620
Ile Arg Leu Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn			
	625	630	635
Phe Met Gly Asn Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro			
	640	645	650
Arg Gly Pro Gln Thr Leu Pro Thr Gly Lys Val Leu Pro Gly Asn			
	655	670	675
Asn Asn Ser Tyr Asp Lys Cys Arg Arg Arg Phe Asp Leu Val Asn			
	680	685	690
Ala Asp Phe Leu Arg Tyr Arg Gly Met Gln Glu Phe Asp Gln Ala			
	695	700	705
Met Gln His Leu Glu Glu Lys Tyr Gly Phe Met Thr Ser Glu His			
	710	715	720
Gln Tyr Val Ser Arg Lys His Glu Glu Asp Lys Val Ile Ile Leu			
	725	730	735
Lys Arg Gly Asp Leu Val Phe Val Phe Asn Phe His Trp Ser Asn			
	740	745	750
Ser Phe Phe Asp Tyr Arg Val Gly Cys Ser Lys Pro Gly Lys Tyr			
	755	760	765
Lys Val Ala Leu Asp Ser Asp Asp Ala Leu Phe Gly Gly Phe Ser			
	770	775	780
Arg Leu Asp His Asp Val Asp Tyr Phe Thr Thr Glu His Pro His			
	785	790	795
Asp Asn Arg Pro Arg Ser Phe Ser Val Tyr Thr Pro Ser Arg Thr			
	800	805	810
Ala Val Val Tyr Ala Leu Thr Glu			
	815		

FIGURE 8

2

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FIGURE 8

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Asp Thr His Tyr Phe His Gly Gly Pro Arg Gly His His Trp Met	425	430	435
Trp Asp Ser Arg Leu Phe Asn Tyr Gly Ser Trp Glu Val Leu Arg	440	445	450
Phe Leu Leu Ser Asn Ala Arg Trp Trp Leu Glu Glu Tyr Lys Phe	455	460	465
Asp Gly Phe Arg Phe Asp Gly Val Thr Ser Met Met Tyr Thr His	470	475	480
His Gly Leu Gln Met Thr Phe Thr Gly Asn Tyr Gly Glu Tyr Phe	485	490	495
Gly Phe Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met Leu Val	500	505	510
Asn Asp Leu Ile His Gly Leu His Pro Asp Ala Val Ser Ile Gly	515	520	525
Glu Asp Val Ser Gly Met Pro Thr Phe Cys Ile Pro Val Pro Asp	530	535	540
Gly Gly Val Gly Leu Asp Tyr Arg Leu His Met Ala Val Ala Asp	545	550	555
Lys Trp Ile Glu Leu Leu Lys Gln Ser Asp Glu Ser Trp Lys Met	560	565	570
Gly Asp Ile Val His Thr Leu Thr Asn Arg Arg Trp Leu Glu Lys	575	580	590
Cys Val Thr Tyr Ala Glu Ser His Asp Gln Ala Leu Val Gly Asp	595	600	605
Lys Thr Ile Ala Phe Trp Leu Met Asp Lys Asp Met Tyr Asp Phe	610	615	620
Met Ala Leu Asp Arg Pro Ser Thr Pro Arg Ile Asp Arg Gly Ile	625	630	635
Ala Leu His Lys Met Ile Arg Leu Val Thr Met Gly Leu Gly Gly	640	645	650
Glu Gly Tyr Leu Asn Phe Met Gly Asn Glu Phe Gly His Pro Glu	655	660	665
Trp Ile Asp Phe Pro Arg Gly Pro Gln Thr Leu Pro Thr Gly Lys	670	680	685
Val Leu Pro Gly Asn Asn Asn Ser Tyr Asp Lys Cys Arg Arg Arg	690	695	700
Phe Asp Leu Gly Asp Ala Asp Phe Leu Arg Tyr His Gly Met Gln	705	710	715
Glu Phe Asp Gln Ala Met Gln His Leu Glu Glu Lys Tyr Gly Phe	720	725	730
Met Thr Ser Glu His Gln Tyr Val Ser Arg Lys His Glu Glu Asp	735	740	745
Lys Val Ile Ile Phe Glu Arg Gly Asp Leu Val Phe Val Phe Asn	750	755	760
Phe His Trp Ser Asn Ser Phe Phe Asp Tyr Arg Val Gly Cys Ser	765	770	775
Arg Pro Gly Lys Tyr Lys Val Ala Leu Asp Ser Asp Asp Ala Leu	780	785	790
Phe Gly Gly Phe Ser Arg Leu Asp His Asp Val Asp Tyr Phe Thr	795	800	805
Thr Glu His Pro His Asp Asn Arg Pro Arg Ser Phe Ser Val Tyr	810	815	820
Thr Pro Ser Arg Thr Ala Val Val Tyr Ala Leu Thr Glu	825	830	

FIGURE 8

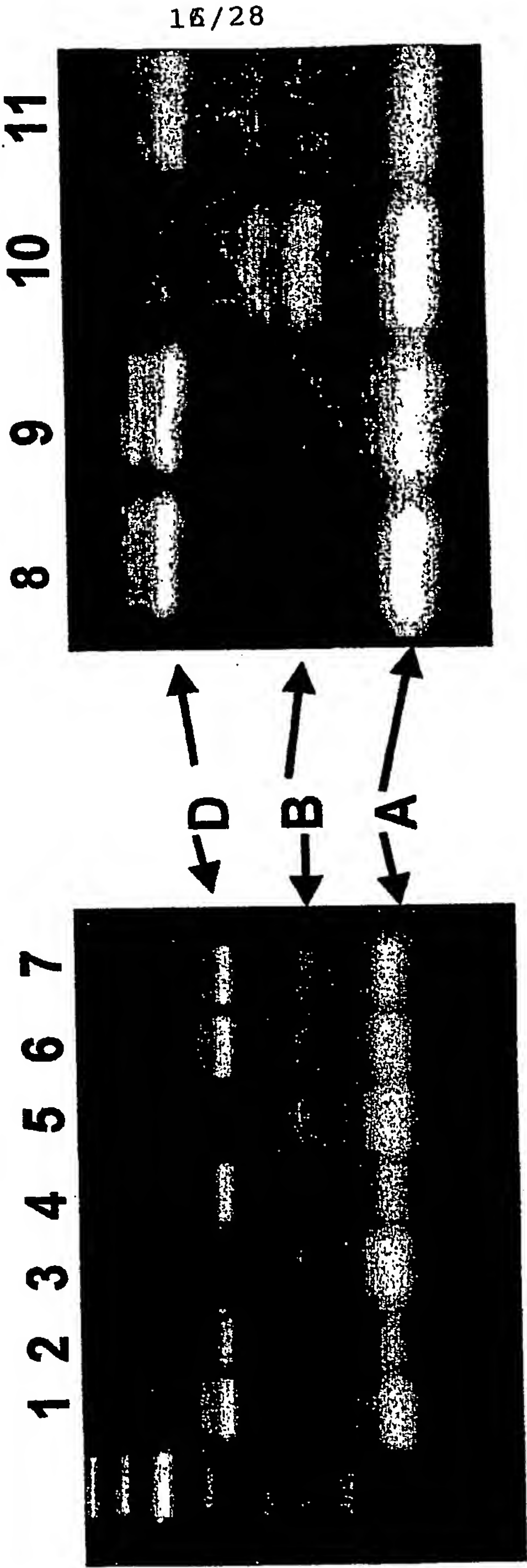


Figure 9

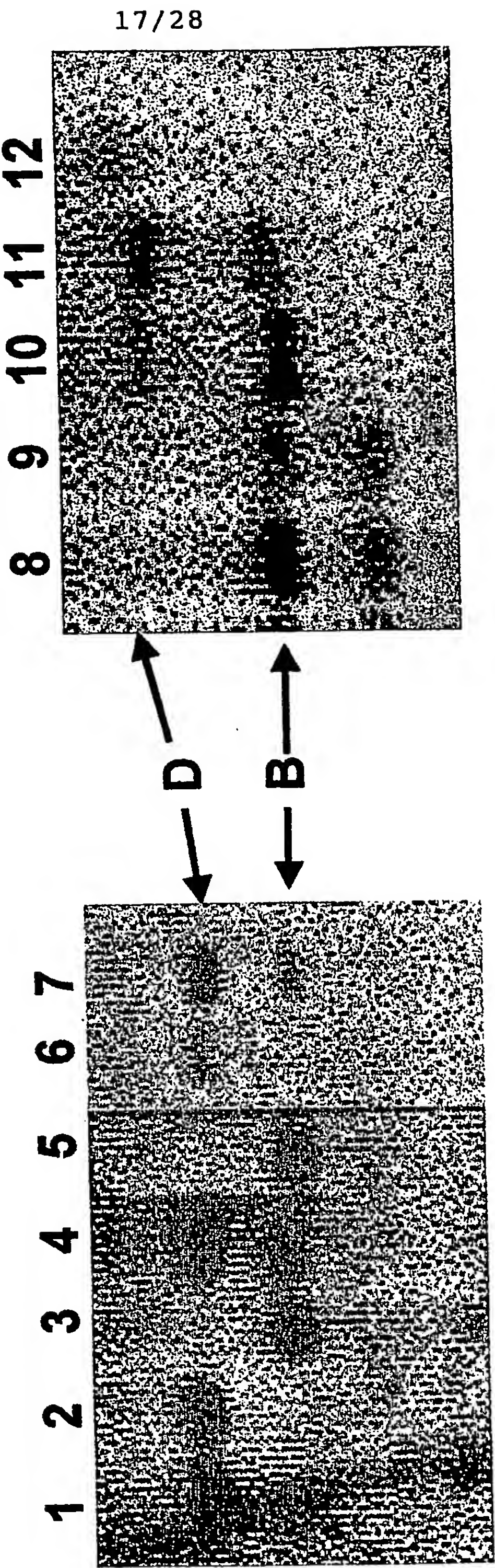


Figure 10

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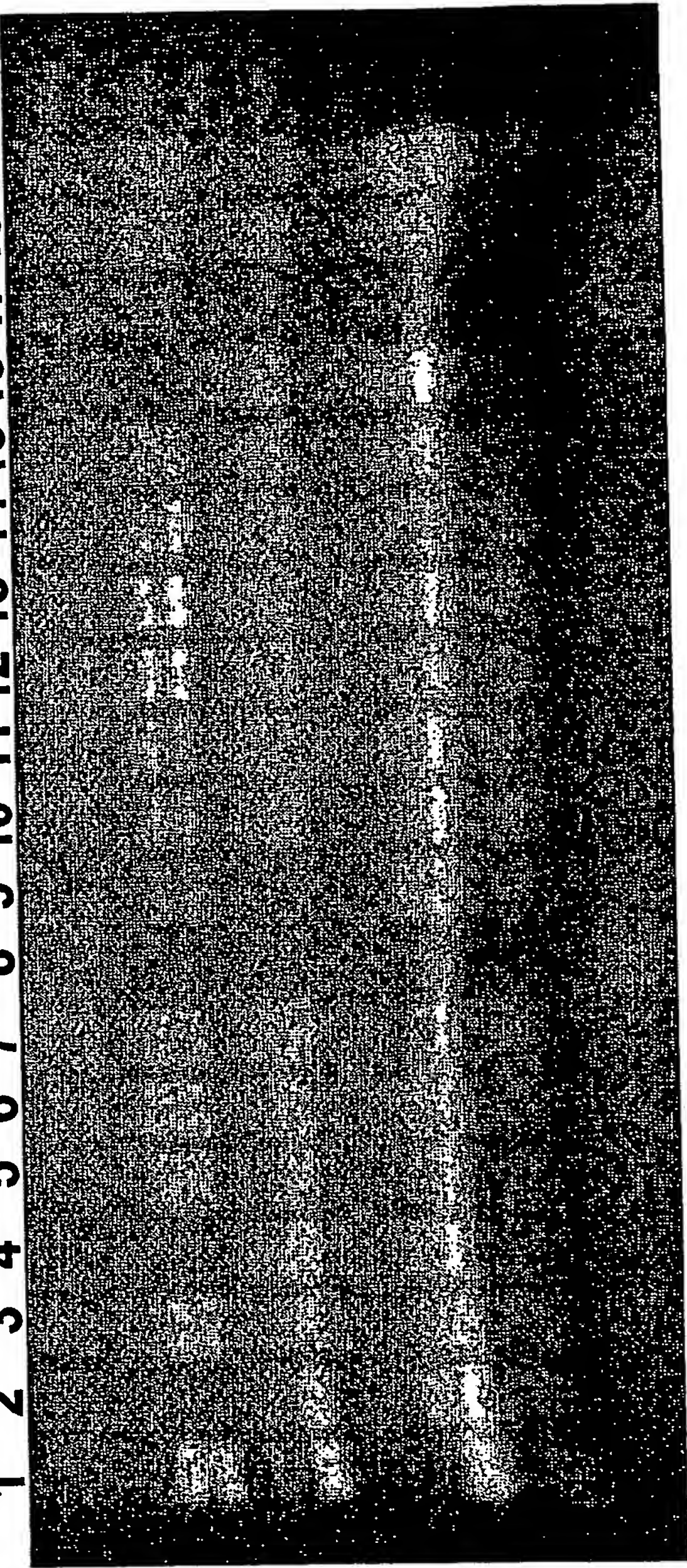


Figure 11

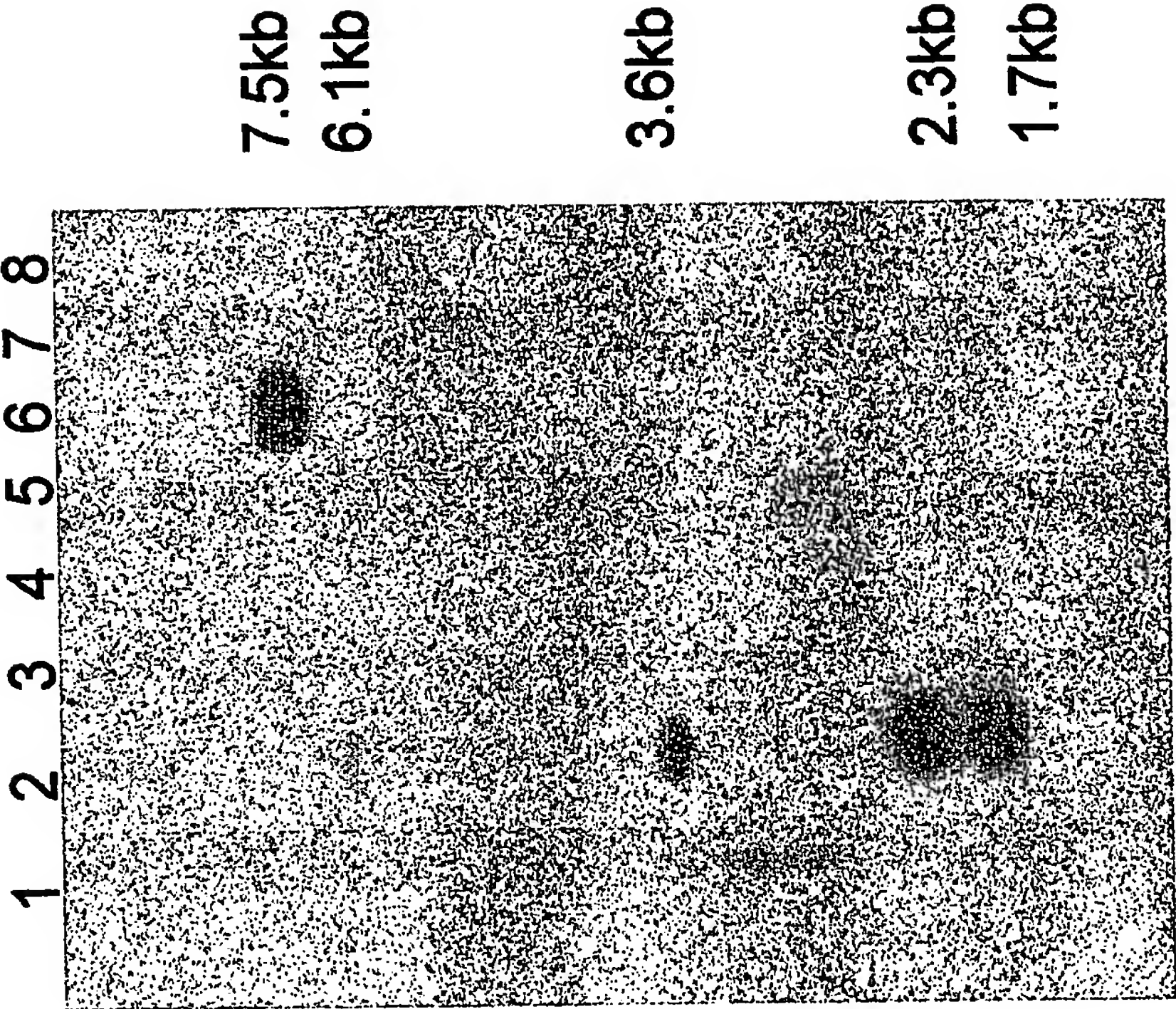
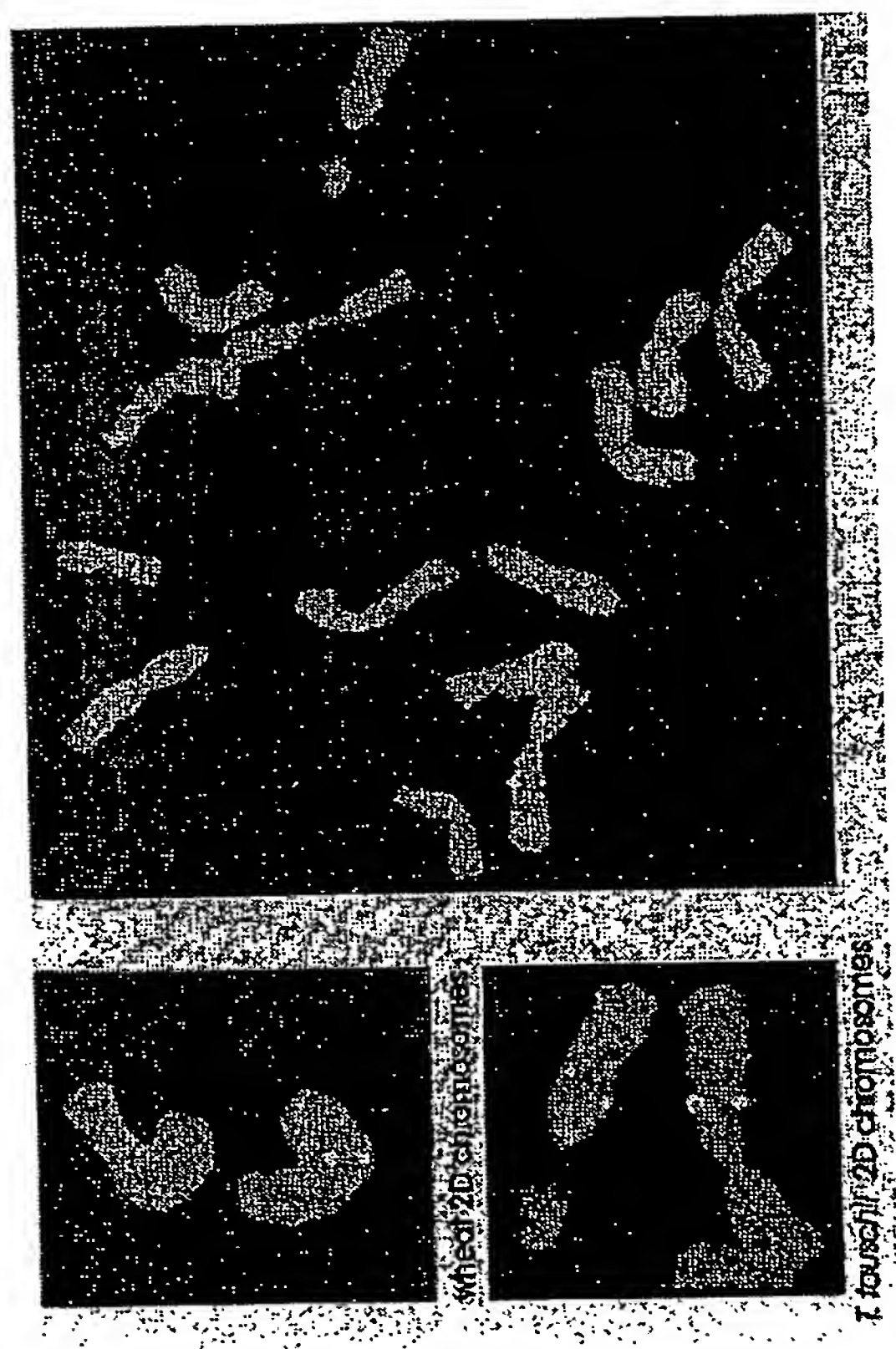
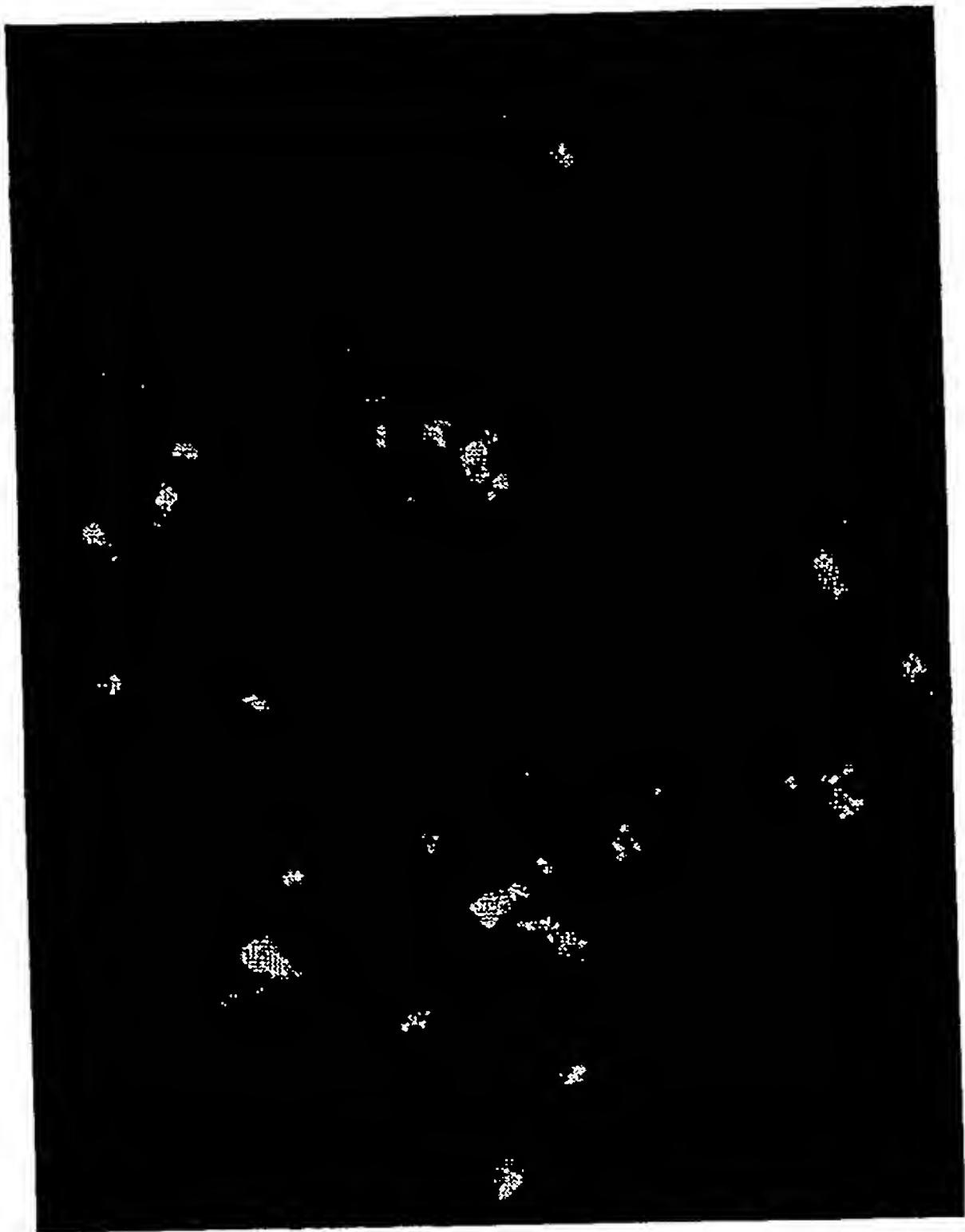


Figure 12



A



B

Figure 13

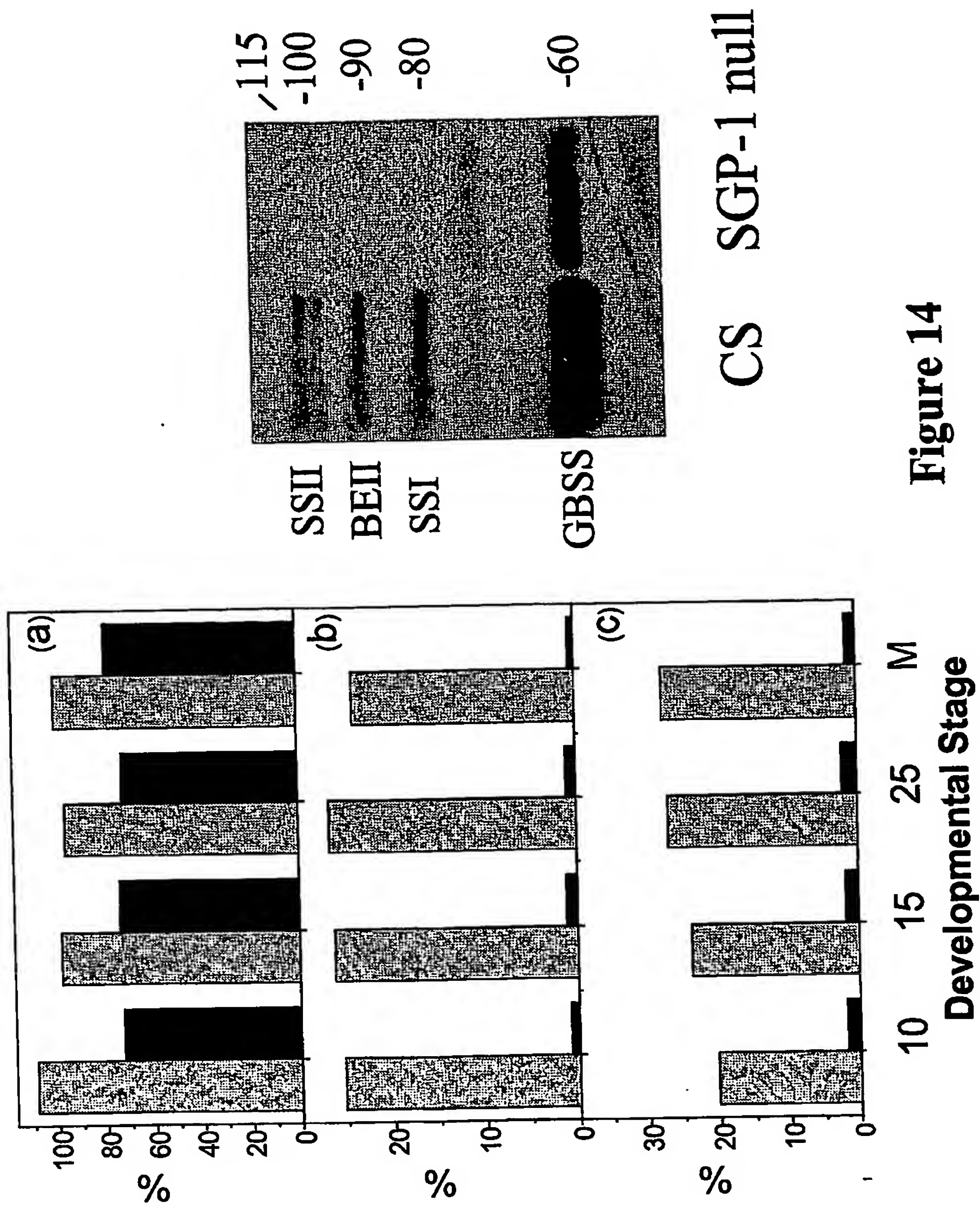


Figure 14

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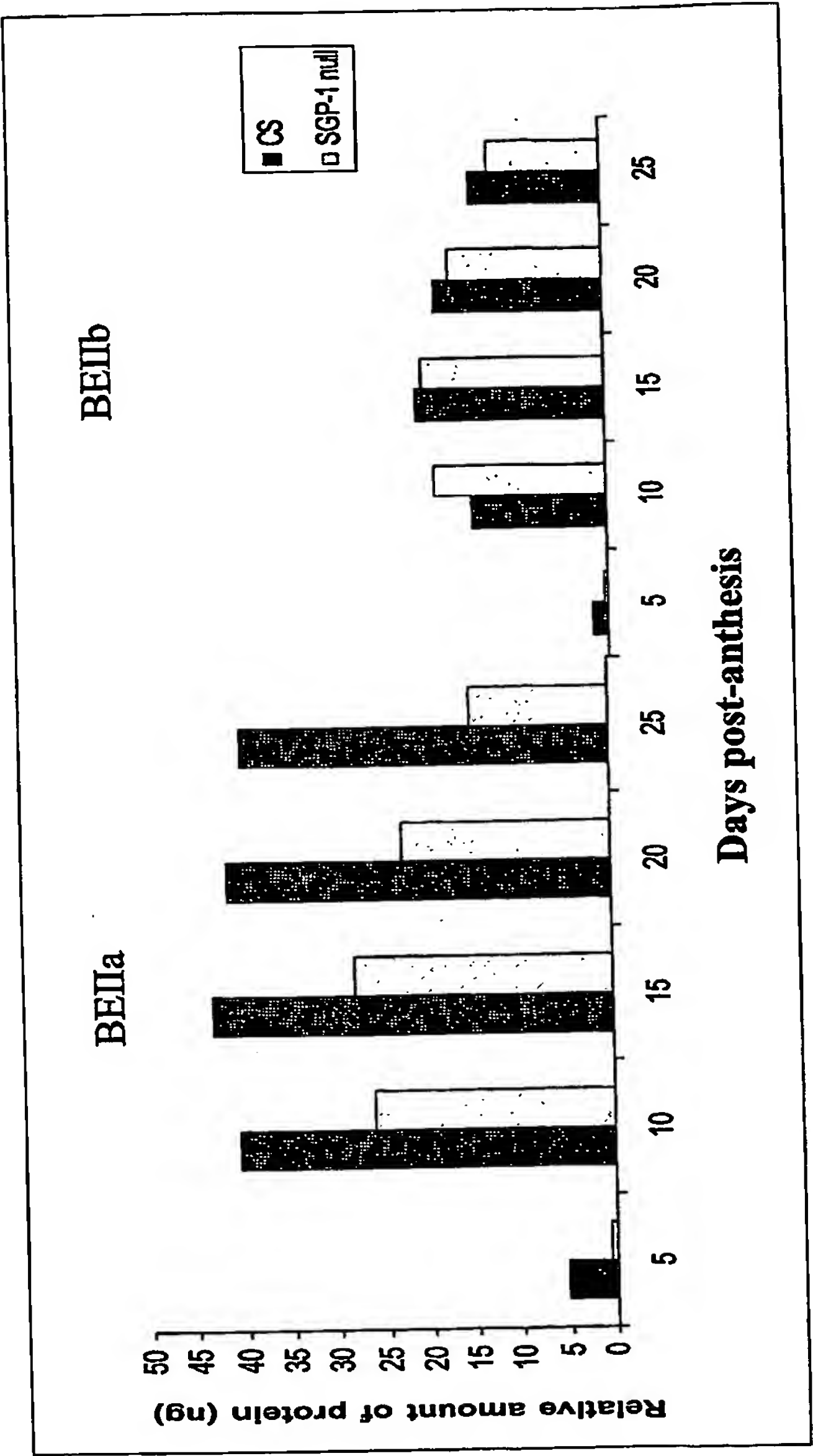


Figure 15

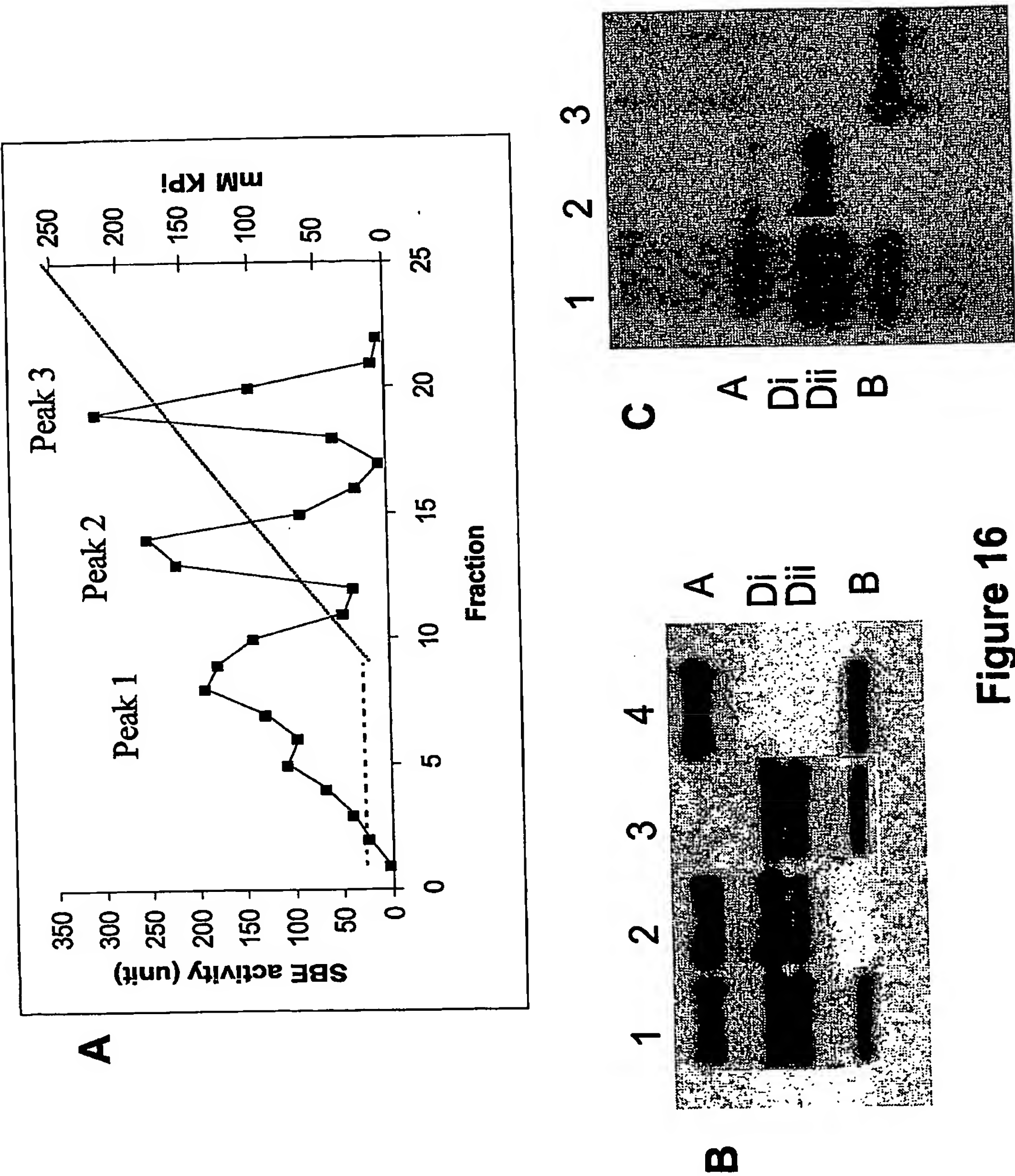




Figure 17

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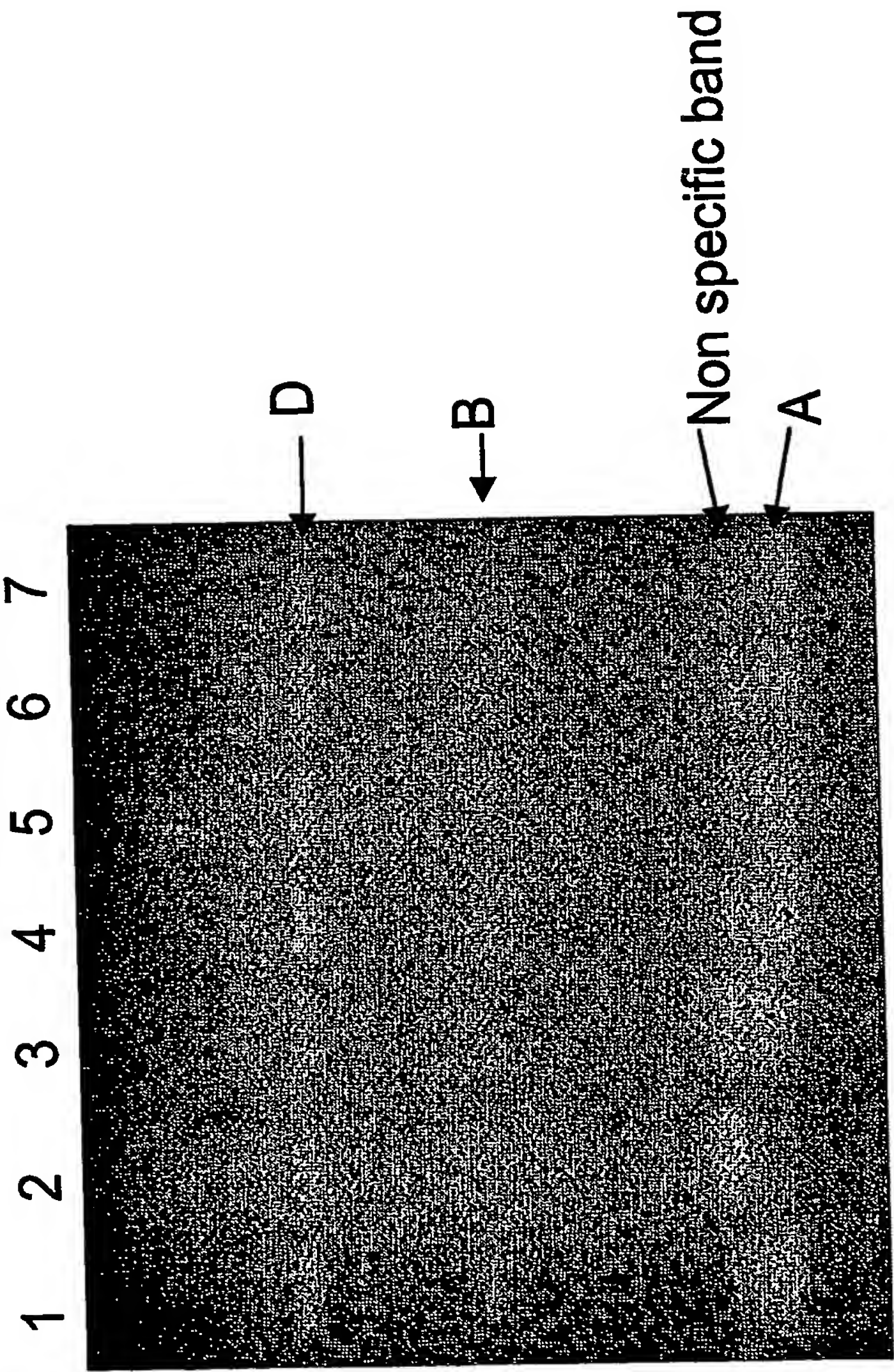


Figure 18

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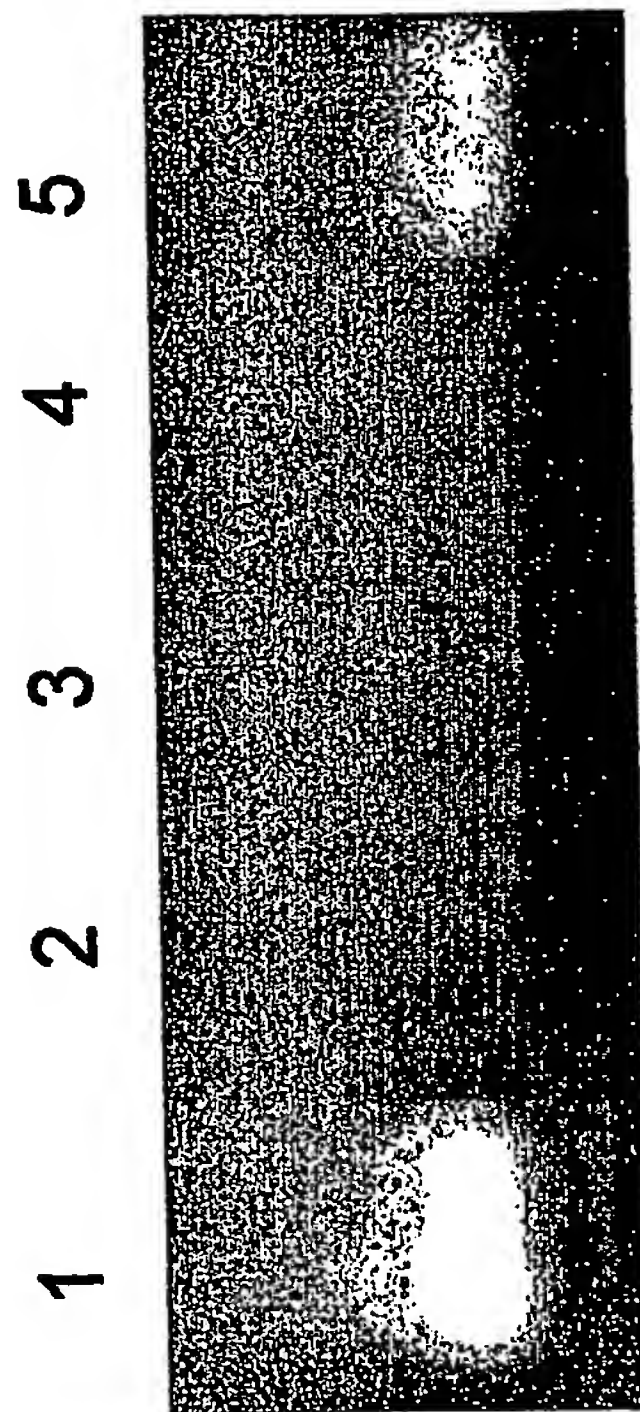
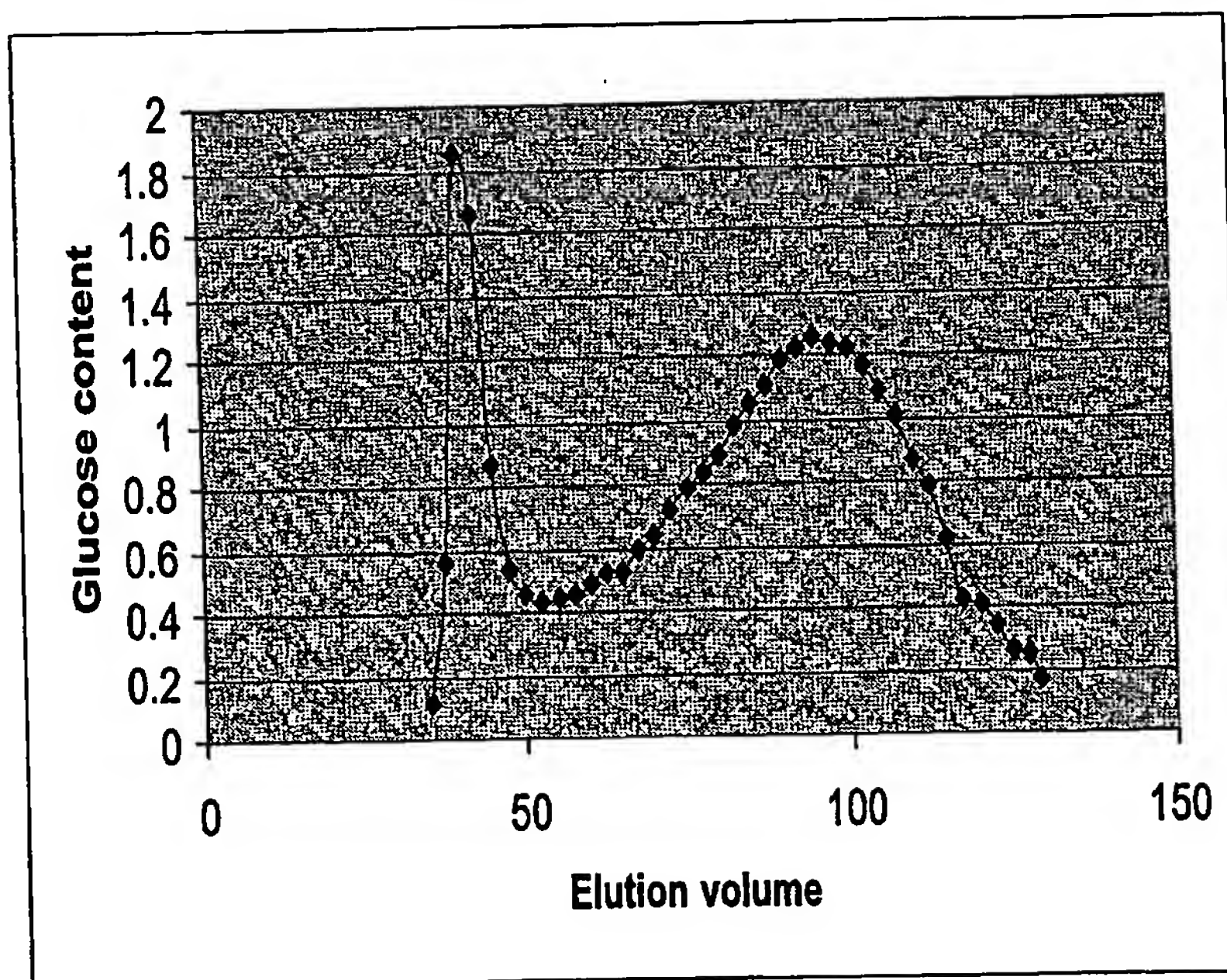
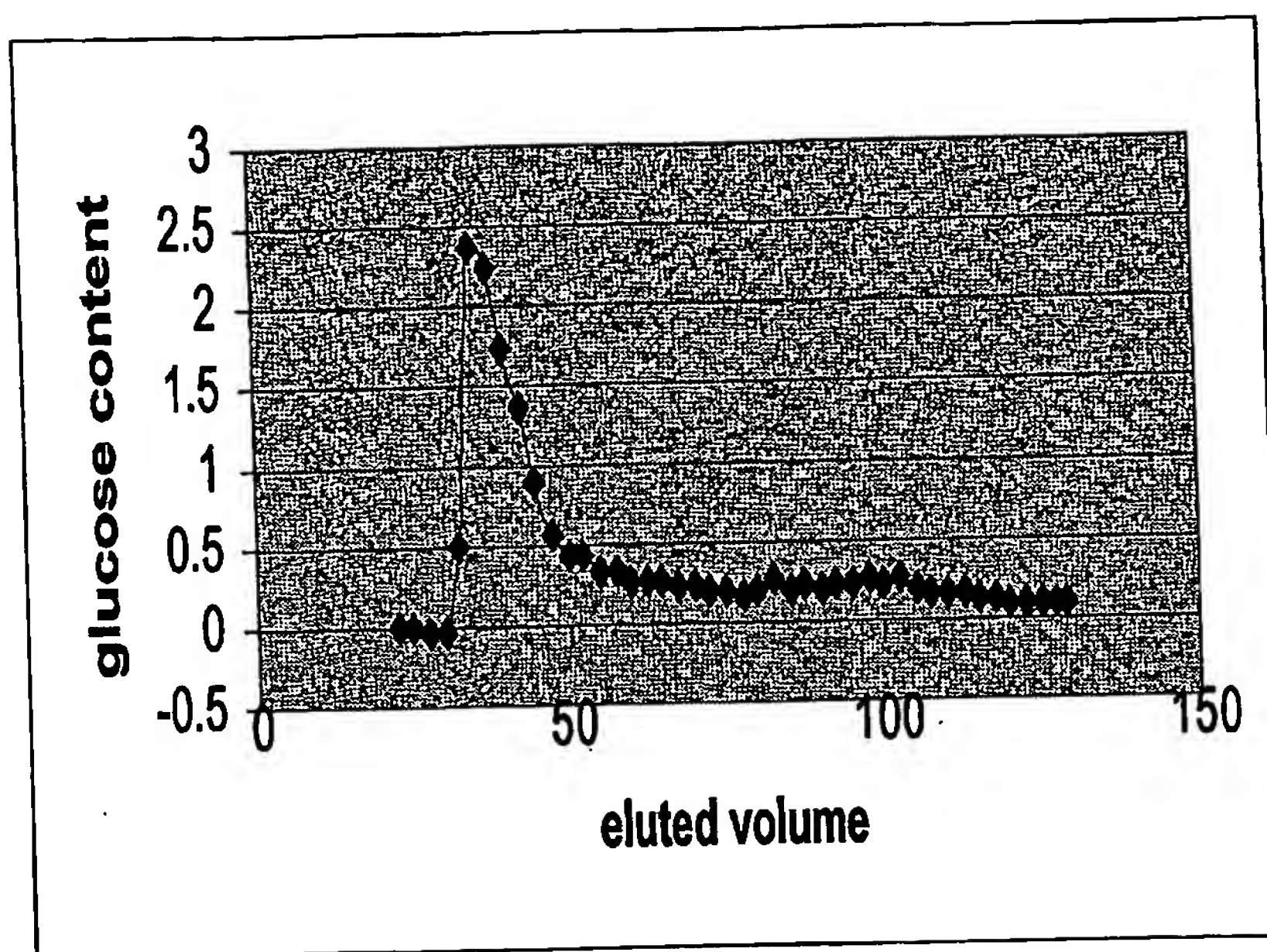


Figure 19

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**Figure 20**

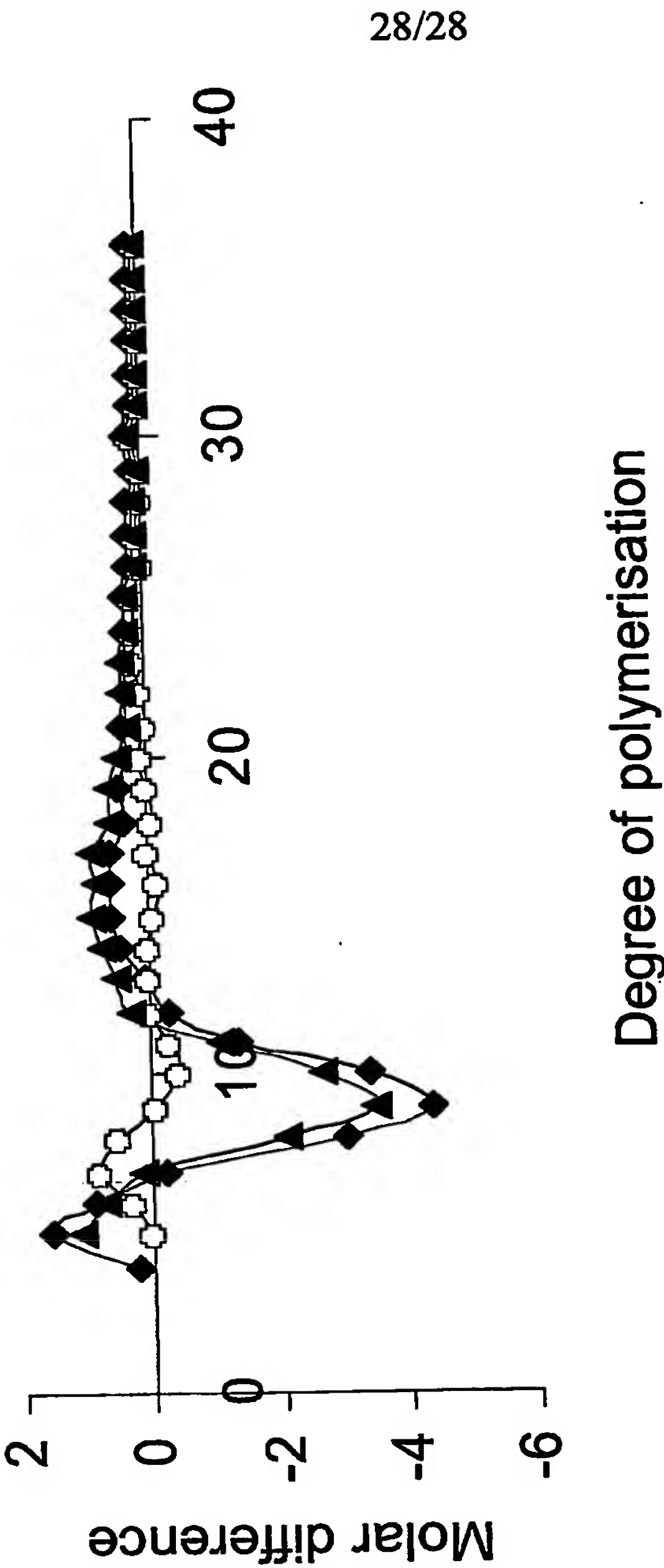


Figure 21

Sequence Listing

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5 GF Group Services Pty Ltd
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containing products with an increase amylose content
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10 <211> 11476
<212> DNA
<213> Aegilops tauschii
<223> wSBRII-D1 gene
<400> 1
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000901

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁷ : C12N 15/52, 15/82, 9/10, A01H 1/00, 5/00, A23L 1/0522 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA, WPIDS, AGRICOLA: wheat, SBEIIa, starch branching enzyme IIa				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
P,A	WO 2003/094600 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 20 November 2003 Entire specification	1-68		
A	WO 2000/015810 A1 (PLANT BREEDING INTERNATIONAL CAMBRIDGE LIMITED) 23 March 2000 Entire specification	1-68		
A	RAHMAN, S. et al., "Comparison of Starch-Branching Enzyme Gene Reveals Evolutionary Relationships Among Isoforms. Characterization of a Gene for Starch-Branching Enzyme IIa from the Wheat D genome Donor <i>Aegilops tauschii</i> ", Plant Physiology, March 2001, Vol. 125, pages 1314-24 Entire article	1-68		
A	SUN, C. et al., "The Two Genes Encoding Starch-Branching Enzymes IIa and IIb Are Differentially Expressed in Barley", Plant Physiology, 1998, Vol. 118, pages 37-49 Entire article	1-68		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search 14 October 2004		Date of mailing of the international search report 2 NOV 2004		
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. (02) 6285 3929		Authorized officer JAMIE TURNER Telephone No : (02) 6283 2071		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000901

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MIAO, H. et al., "Evaluation and characterization of an endosperm-specific sbell_a promoter in wheat", Chinese Science Bulletin, March 2004, Vol. 49, No. 6, pages 579-85</p> <p>Entire article</p>	1-68

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000901

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 11-25, 31-32, 50-65, 67-68 (all in part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 1, 11-25, 31-32, 50-65, 67-68 (all in part) are not limited to the technical features of the invention, (which is considered to be the manipulation of SBEIIa protein, enzyme activity, or gene expression to produce wheat grain with starch having a reduced proportion of amylose).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2004/000901

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	03094600	US	2004060083		
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